Natural products for malaria vector control: flora, fish and fungi

Annabel F. V. Howard

Thesis committee

Thesis supervisors

Prof. dr. ir. W. Takken
Personal chair at the Laboratory of Entomology,
Wageningen University

Prof. dr. M. Dicke
Professor of Entomology,
Wageningen University

Thesis co-supervisor

Dr. J.J. Githure
Head of Human Health,
International Centre of Insect Physiology and Ecology, Kenya

Other members

Prof. dr. ir. R. Rabbinge
Wageningen University

Dr. W.J. de Kogel
Plant Research International, Wageningen University and Research Centre

Prof. dr. E. van Donk
Netherlands Institute of Ecology, Maarssen

Dr. W.J. Ravensberg
Koppert B. V., Berkel en Rodenrijs

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Annabel F. V. Howard

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Abbreviations

ACT	Artemisinin-based combination therapy
AZA	Azadirachtin
BC	*Beauveria bassiana* window, control bednet
BP	*Beauveria bassiana* window, permethrin bednet
Bti	*Bacillus thuringiensis* var. *israelensis*
CC	Control window, control bednet
CI	Confidence interval
COMBI	Communication for Behavioural Impact
CP	Control window, permethrin bednet
CREC	Centre de Recherche Entomologique de Cotonou
DDT	Dichlorodiphenyltrichloroethane
DT	Dispersible tablet
df	Degrees of freedom
EC	Emulsion concentrate
EIP	Extrinsic incubation period
EIR	Entomological inoculation rate
FD	Fisheries Department
GC	Gonotrophic cycle
GST	Glutathione-*S*-transferase
HBI	Human blood indices
HPLC	High performance liquid chromatography
HR	Hazard Ratios
icipe	International Centre of Insect Physiology and Ecology
IE_{50}	The concentration that causes 50% inhibition of emergence
IE_{90}	The concentration that causes 90% inhibition of emergence
IEC	Information Education and Communication
IGR	Insect growth regulators
IPM	Integrated pest management
IRS	Indoor residual spraying
ITN	Insecticide-treated bednet
ITU	International toxic units
IVM	Integrated vector management

kdr	Knockdown resistance
KEMRI	Kenya Medical Research Institute
KNAW	Royal Dutch Academy of Arts and Sciences
L:D	Light : Dark
LI	1st instar mosquito larva
LII	2nd instar mosquito larva
LIII	3rd instar mosquito larva
LIV	4th instar mosquito larva
LSHTM	London School of Hygiene and Tropical Medicine
MC	*Metarhizium anisopliae* window, control bednet
MDG	Millennium Development Goals
MFO	Mixed function oxidase
MP	*Metarhizium anisopliae* window, permethrin bednet
N	Number
NCP	Neem seed cake powder
NWM	Not well maintained
OR	Odds Ratios
PE&RC	Production Ecology and Resource Conservation
RH	Relative humidity
SDA	Sabouraud Dextrose Agar
SE	Standard error
SKK	*Anopheles gambiae s.s.* Suakoko strain
s.l.	*Sensu lato*
SNK	Student-Newman-Keuls
Spp.	Species (plural)
s.s.	*Sensu stricto*
UN	United Nations
UV	Ultraviolet light
VKPER	*Anopheles gambiae s.s.* Valley de Kou pyrethroid-resistant strain
WHA	World Health Assembly
WHO	World Health Organisation
WHOPES	World Health Organisation Pesticide Evaluation Scheme
WM	Well maintained

Abstract

Introduction

Despite international organisations providing much focus over the past 10 years, malaria is still killing vast numbers of Africans, especially children. It is agreed that malaria can only be successfully controlled by using different control tools simultaneously in the spirit of integrated vector management (IVM), and that African communities will need to become more directly involved in mosquito control (**Chapter 2**). Using mosquito control tools in a way that requires almost no technical equipment or knowledge will open them up to the rural communities that are best placed to deploy them. In addition, widespread insecticide resistance is reducing the ability of insecticide-based tools to control mosquitoes. For these reasons, biological control and other natural mosquito control methods are being researched by many institutions. Several potential natural control tools are readily available in sub-Saharan Africa. If these tools prove effective and become operational, then it is possible that they will be sustainable because communities can intentionally produce the biological agents themselves, bringing a source of money to rural communities. This would be especially important in areas where infrastructure is poorly developed, and repeat applications of chemical control tools are not easily made. This thesis was designed to test the feasibility and effectiveness of a variety of natural products against both larval and adult malaria vector mosquitoes using low-tech methods in laboratory and field trials.

Part I: Flora

Azadirachta indica A. Juss (Meliaceae) (the neem tree) was chosen due to the already proved mosquitocidal properties, and its ready availability in Africa. We wanted to use neem in a way that could easily be deployed in resource-poor rural

areas. Laboratory studies were conducted to examine the larvicidal and pupicidal properties of a crude aqueous extract of neem wood against the principle African malaria vector, *Anopheles gambiae* Giles *s.s.* (Diptera: Culicidae) (**Chapter 3**). The results indicate that even a relatively low dose of 0.15 grams of dried neem wood in 1 litre of water was able to inhibit the emergence of 90% of mosquito adults when larvae were exposed during their first three larval instars. Even for the fourth (last) larval instar, just 0.6 g/l was required to prevent 90% emergence. Furthermore, neem-exposed larvae exhibited significantly increased development times when compared to the controls. Pupae were also killed by the aqueous neem extracts, and were subject to neem-induced emergence abnormalities, but the concentrations required to kill pupae were much higher than for larvae and not likely to be used operationally. High performance liquid chromatography (HPLC) analysis identified several polar constituents in the aqueous neem extracts including nimbin and salannin. However, azadirachtin was not present in significant amounts. The effect of this extract on the oviposition behaviour of adult female *An. gambiae s.s.* mosquitoes was then monitored (**Chapter 4**). The oviposition results show that when using 0.1 g/l of the crude aqueous neem extract, significantly more mosquitoes laid their eggs when compared to mosquitoes exposed to the control treatment. For the doses 10x and 100x higher, the same proportion of mosquitoes laid their eggs as in the control, indicating that even at much higher doses than required for successful larval control, female oviposition will not be detrimentally affected.

Part II: Fish

Larvivorous fish have previously been shown to effectively control mosquito numbers. Therefore, a census was carried out to examine the current status of fish farming in western Kenya (**Chapter 5**). Working with the Kenyan Fisheries Department we found that while fish farming is a favoured activity, 30% of the 261

ponds found did not contain fish. These “abandoned” ponds had significantly more *An. gambiae s.l.*, *Anopheles funestus* Giles and culicine mosquitoes when compared to the ponds that still contained fish. Furthermore, *An. gambiae s.l.* was proportionally more abundant in the abandoned ponds when compared to the other mosquito types. Surprisingly, vegetation did not significantly affect mosquito distribution. Following our study, demand for fish to restock abandoned ponds increased by 67% when compared to the previous year. The overwhelming majority of fish being farmed in our census area were fish of the tilapiine subfamily. Given this finding, we set up a small-scale field experiment to study the larvivorous potential of the tilapiine fish *Oreochromis niloticus* L. (Perciformes: Cichlidae) (**Chapter 6**). Taking daily measurements of mosquito numbers, we found that immediately after fish introduction, the density of mosquitoes in the treated ponds dropped in comparison to the increase in the control pond. After 15 weeks, anopheline numbers had decreased by >94% in the ponds containing the fish, and we found that fish were able to sustainably control mosquitoes for at least 6 months, when our study finished. It is concluded that this type of fish could be an effective and sustainable way to control mosquito numbers in rural western Kenya. Furthermore, this fish provides a source of much needed income and protein to rural African communities.

Part III: Fungi

For the control of mosquito adults using natural products, entomopathogenic fungi hold the most promise. In this thesis the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* were separately suspended in mineral oil and applied to polyester netting. A laboratory experiment was then conducted to investigate the fungal susceptibility of insecticide-susceptible and insecticide-resistant strains of *An. gambiae s.s.*. In addition, fungal conidial viability was tested at various time points after application onto polyester netting (**Chapter 7**). Whilst

both mosquito strains were susceptible to both species of fungal infection, the pyrethroid-resistant *An. gambiae s.s.* VKPER strain was significantly more susceptible than the insecticide-susceptible SKK strain, dying more quickly. Conidial viability was significantly lower for both species after application onto the polyester netting when compared to the viability in suspension. However, the ability of the treated netting to infect and kill mosquitoes was not significantly diminished over the one week trial period. Given the finding that fungal-treated polyester netting could infect and kill mosquitoes, an experimental hut field trial was conducted in Benin, West Africa, to investigate the effect of fungal treatment on blood feeding behaviour and survival of wild insecticide-resistant mosquitoes. Benin was chosen due to the presence of multi-insecticide-resistant mosquito populations that are threatening the effectiveness of current vector control. We used fungal-treated netting to infect mosquitoes entering the hut windows, and either an untreated or insecticide-treated bednet was placed into each hut to examine how the entomopathogenic fungi would work with current control tools (**Chapter 8**). Only enough *Culex quinquefasciatus* Say (Diptera: Culicidae) mosquitoes were collected from the huts for accurate analysis. Our study was the first to monitor the effect of entomopathogenic fungi on blood feeding of wild mosquitoes. We found that the *B. bassiana* treatments caused significant and instantaneous reductions in blood feeding. No significant effect of the fungi on mosquito mortality was found. Conidial viability of *B. bassiana* and *M. anisopliae* was found to decrease rapidly under field conditions.

Conclusions

This thesis used several different experimental techniques to examine the potential of three natural products to control mosquitoes. For the flora, it was found that even a small amount of neem wood in water would control mosquitoes (**Chapter 3**), and at this and higher doses, the oviposition behaviour was not adversely affected

(**Chapter 4**). Neem trees are readily available in many areas of Africa, and promising field trials indicate that the use of this tree species should be incorporated into malaria control trials.

This thesis reports that edible native African fish can be effective at controlling mosquitoes (**Chapter 6**), but if fish farming is abandoned and the ponds not filled in, then they can allow large numbers of the most effective malaria vectors to breed (**Chapter 5**). Fish have been successfully used for malaria vector control in many countries and this could be rolled out across appropriate areas of Africa, as long as it is accompanied with adequate education about the dangers of abandoned ponds.

We found that insecticide-resistant mosquitoes were more susceptible to fungal infection than the insecticide-susceptible strain. Under field conditions fungi were able to prevent blood feeding but did not cause significant mortality in the wild-caught mosquitoes. Although entomopathogenic fungi produce high levels of mortality in laboratory settings, (**Chapter 7**), their use under field conditions still has a long way to go and is not yet at the operational stage. Although the results found in this thesis are encouraging for the use of fungi in African situations (**Chapter 8**), further work should be carried out to maximise fungal persistence under field conditions.

The current emphasis is on IVM for malaria control (**Chapter 2**), and focus is turning to biological control tools that can help manage insecticide-resistant populations. With this in mind, the natural products investigated in this thesis have produced encouraging results that show they have the potential to be integrated into malaria control strategies. Furthermore, flora and fish are readily available in the areas where they are most required, and could be used almost immediately to help reduce mosquito numbers and correspondingly, malaria disease transmission.

Chapter 1

General Introduction

1.1 Malaria: disease dynamics and transmission

Malaria is a predictable, preventable and treatable disease that worldwide still kills one child almost every 30 seconds. It is one of the most important tropical parasitic diseases in the world and is caused by obligate intracellular protistan parasites. Five species of these *Plasmodium* parasites infect humans. These are *P. vivax, P. ovale, P. malariae, P. knowlesi* and *P. falciparum* (Knell 1991, White 2008). The latter is the most severe form and can lead to coma and death within a few days for particularly susceptible people. Other symptoms include fever, chills, anaemia, splenomegaly, vomiting and diarrhoea. Human malaria is transmitted by *Anopheles* spp. (Diptera: Culicidae) mosquitoes.

Although malaria transmission is centred on the tropics, it can also be found in sub-tropical areas (Figure 1.1) and globally it is estimated that half of the world's population is at risk of malaria (Hay et al. 2004, World Health Organisation 2009). The threat and burden of malaria is not equally distributed; there is a disproportionate burden of disease in the most resource-poor countries, especially

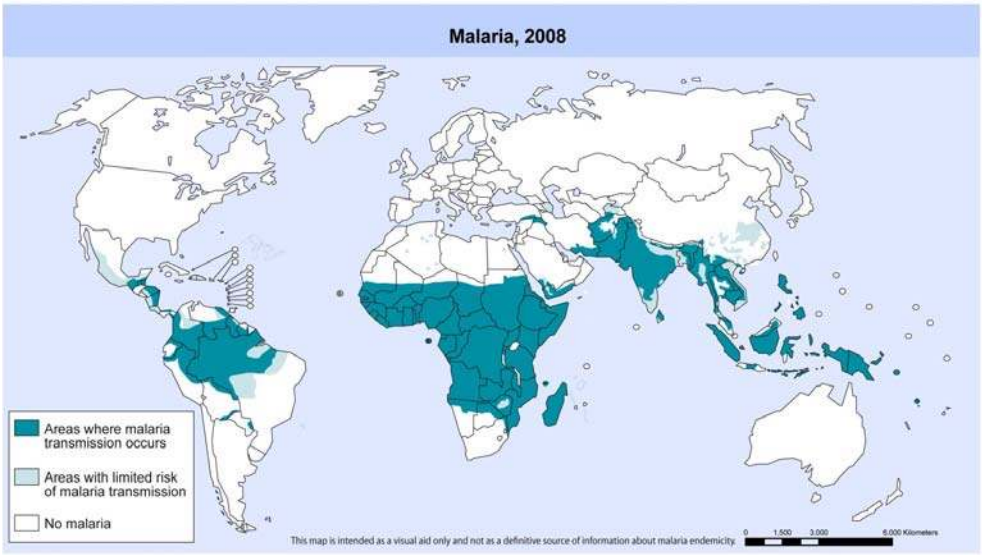

Figure 1.1. Global distribution of malaria (Source WHO, www.who.org)

in sub-Saharan Africa. Even within African countries malaria is disproportionately distributed between rural and urban communities (see **section 1.2**). The entomological inoculation rate (EIR) is the number of mosquito bites infected with malaria that any one person is expected to receive in one day and is a measure for actual transmission rates. The EIR varies between different areas mainly due to differences in the local climate, and the capacity and species distribution of the local mosquito vectors. For example, annual estimates of EIR range from 3.6 infected bites per person per year in Mali to 814 bites per person per year in Equatorial Guinea (Kelly-Hope and McKenzie 2009).

It is estimated that in 2008 there were 243 million cases of malaria and 863,000 deaths (World Health Organisation 2009). Although malaria is endemic in 108 countries, 99% of the population at risk in the African region live in 35 high-burden countries. It is estimated that 85% of all cases and 89% of all deaths caused by malaria in 2008 occurred in Africa (World Health Organisation 2009). Mostly, children under the age of five, pregnant women, and non-immune individuals are at risk of dying of malaria. Children under the age of five are more likely to die of malaria because they have not built up an adequate immune response. Pregnant women are not only more likely to get infected with malaria than those who are not pregnant; they are also more liable to have severe malaria. Amongst other non-lethal effects in pregnant women, malaria infections can cause both the mother and foetus to die (Lagerberg 2008). This risk is especially true for those experiencing their first pregnancy.

As well as the physical toll of malaria, this parasitic disease also imposes a financial burden. Poor levels of investment and weak economic growth in some areas of Africa have been blamed in part on the presence of malaria (Hay et al. 2000, Sachs and Malaney 2002) with gross domestic product being five times lower in countries that have malaria when compared to non-malarious countries (Gallup and Sachs 2001). On a more personal scale, people infected with malaria can lose time at work if they themselves are ill or if they need to take a child to the

health centre. As well as losing money from lost time at work, in some areas they still need to pay for malaria prevention, treatment and when necessary, funerals.

The World Health Organisation (WHO) recommends that all suspected malaria cases be diagnosed either using rapid diagnostic tests or microscopy of blood films. However, in the 18 countries that reported on this in 2008, just 22% of people were undergoing diagnostic testing (World Health Organisation 2009). Similarly, WHO recommends that all confirmed cases of uncomplicated *P. falciparum* malaria are treated with artemisinin-based combination therapy (ACT) (other species of malaria parasites require different treatment regimes). Although the policy in many countries is to use ACTs, unfortunately, not all these countries have managed to deploy their use. Five ACTs are recommended to treat uncomplicated *P. falciparum* malaria and they are being used in 77 of the 81 countries where this species is endemic (World Health Organisation 2009). However, just five countries reported distributing enough ACTs to treat all reported malaria cases in 2008 (World Health Organisation 2009). Even in countries where the policy is to use ACTs, in reality the old drugs like chloroquine are still being used (Tipke et al. 2009). As with many other anti-malarial drugs, resistance to artemisinin derivatives has already emerged on the Thai-Cambodian border (Wongsrichanalai and Meshnick 2008) and the failure rate of ACTs is rising in this area (World Health Organisation 2009). A major cause of parasite resistance to artemisinins is continued artemisinin monotherapy in many countries. Although WHO has called for this practise to stop, currently just 44 countries have banned the use of artemisinin monotherapies (World Health Organisation 2009). Despite the low coverage rates and emerging resistance, there is evidence that ACTs have been effective in lowering death rates and parasite reservoirs in a number of African countries (Okell et al. 2008, Barnes et al. 2009).

There is strong political will within the global community to tackle, and in some places eradicate, malaria. By the end of 2010 the aim of the United Nations (UN) (through the Millennium Development Goals (MDG)), the World Health Assembly

(WHA) and WHO is that 80% of people at risk of malaria will be sleeping under an insecticide-treated bednet (ITN), have access to appropriate antimalarial medicine and/or have indoor residual spraying (IRS) where logistically possible. The final goal is that 80% of pregnant women in moderate and high transmission areas will have access to intermittent preventive treatment (World Health Organisation 2009).

In line with these goals, it is hoped that the number of malaria cases and deaths recorded at the end of 2010 will be <50% of the number recorded in 2000 (World Health Organisation 2009). Although ITN, ACT and IRS coverage is still below target levels (Geissbuhler et al. 2009, Matovu et al. 2009, World Health Organisation 2009), it appears that malaria is being successfully controlled in many countries, with large (>50%) reductions in malaria cases in 38 countries, although 29 of these were outside Africa. African countries with high transmission rates that achieved this reduction included Eritrea, Rwanda, Zambia and São Tomé and Principle (World Health Organisation 2009).

1.2 A comparison of urban and rural African communities with reference to malaria

There is a disproportionate malarial disease burden among African communities. People living in rural Africa on the whole suffer more health problems than those in urban environments (Hay et al. 2005). Rural communities have to cope with higher infant and child mortality rates and lower nutritional levels (Hay et al. 2005). Similarly, in general, people living in urban areas are on the whole better off than rural communities (Matovu et al. 2009). In terms of health care provision, urban communities have better access to health facilities (Noor et al. 2003, Hay et al. 2005), were twice as likely to have modern medicines in their home when compared to rural communities (Tipke et al. 2009) and were much more likely to own an ITN (Matovu et al. 2009). A recent study showed that for rural households just 10% of children under the age of five slept under an ITN compared to 47% of

urban children (Matovu et al. 2009).

In addition, rural houses have more malaria vectors in them than urban houses, with the risk of the most efficient African malaria vector, *Anopheles gambiae* Giles *sensu lato*, being present in an urban house 89% lower than in a rural house (Kirby et al. 2008). This may be due to the finding that mud brick houses and those with open eaves and thatch roofs had more mosquitoes in them when compared to concrete houses and those with closed eaves or metal roofs (Kirby et al. 2008). This is unsurprising as mud houses with open eaves offer more access points to mosquitoes than concrete houses with closed eaves. Mud houses are typically found in rural resource-poor African communities and less so in urban areas. Another reason for more mosquitoes being found in rural areas is that there are more anopheline-friendly larval habitats in rural compared to urban areas. Given the higher abundance of malaria vectors in rural areas (Kirby et al. 2008), and the lack of access to health care (Hay et al. 2005), it is no surprise that rural areas typically have higher EIRs (Hay et al. 2005, Kelly-Hope and McKenzie 2009).

In many malaria endemic areas in Africa, people simply cannot afford to pay for malaria prevention, diagnosis and treatment. A study in Burkina Faso found that as the level of poverty increased, the likelihood of finding modern medicines in the home decreased (Tipke et al. 2009). Just 52 of the 108 countries endemic for malaria have a policy to offer free ACTs to children <5 years old (World Health Organisation 2009). Even when something is policy it does not always mean that the medicines are available at the rural level in all areas of the country. And even if the medicines are in every health facility in the country, rural communities in general have less access to health facilities (and consequently ACTs (see Mutabingwa (2005)) than those in urban areas; one meta-analysis found that the median distance to the nearest health facility was 47.6 km for rural people (Hay et al. 2005). In Burkina Faso as distance increased from the health facility, the likelihood of finding modern medicine in the house decreased (Tipke et al. 2009).

1.3 The malaria parasite life cycle

The lifecycle of the *Plasmodium* parasite can be seen in Figure 1.2. Starting from the left of this figure and moving in a clockwise direction, when a female *Anopheles* mosquito takes a blood meal from an infected human she may ingest the male and female gametocytes (as well as the asexual merozoites and schizonts). Inside her gut the male gametocytes undergo a process called exflagellation. This will only take place if certain conditions are present. These include a drop in temperature, an increase in pH and the presence of xanthurenic acid (previously called the gametocyte activating factor) (Billker et al. 1998). Exflagellation is a nuclear division that produces eight haploid motile male gametes and occurs 8-15 minutes after the blood meal is ingested. Meanwhile, the female gametocyte develops into the macrogamete. When the male and female gametes fuse they form a zygote; this matures into an ookinete within a few hours (Knell 1991).

The ookinete is an invasive stage that passes through the gut wall causing localised cell death in the mosquito (Han et al. 2000). When it contacts the basal lamina of the midgut the ookinete develops into an oocyst, which remains fixed at the point of development under the basal lamina. The oocyst (stage 1 of sporogony in Figure 1.2) is a spherical body in which the sporozoites are produced. The sporozoites (typically 9-16.5 μm in length and 0.4-2.7 μm in width) bud off from the central sporoblastoid body of the oocyst (Menard et al. 1997) around 6-9 days post infection (Sherman 1998). Sporozoite maturation is dependent on temperature and when the sporozoites are finally mature, the oocyst bursts releasing thousands of sporozoites into the haemolymph of the mosquito. These travel until they contact and invade the mosquito salivary glands. The median and distal lateral lobes of the glands are the areas preferentially invaded. Sporozoites enter the space between the basal lamina and the basal plasma membrane before invading the cells. Inside the cells they are either found within vacuoles or free in the cytoplasm. They then enter the secretory cavity (invading only the apical plasma membrane) and are again either free in the cytoplasm or surrounded by a vacuole (Sherman 1998).

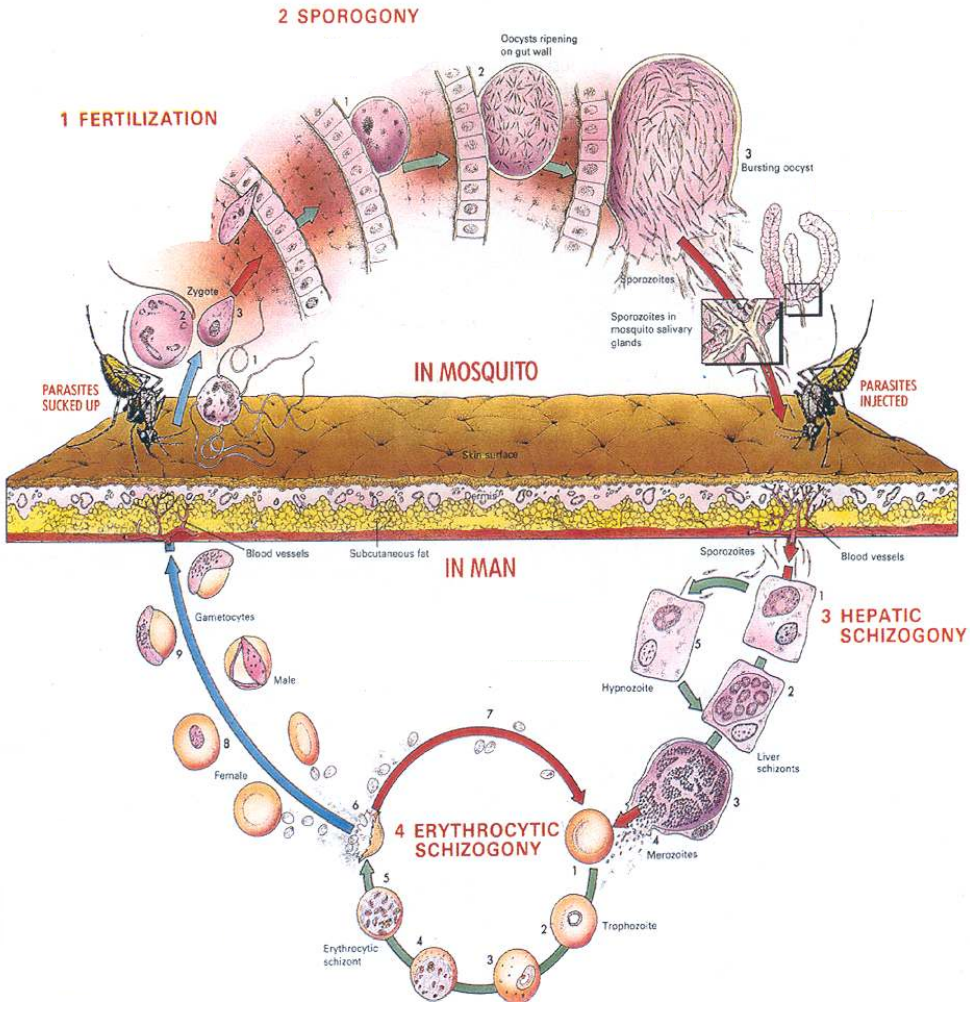

Figure 1.2. *Plasmodium* lifecycle (from Knell 1991)

Malaria sporozoites invade the salivary glands so that they can be 'injected' into the vertebrate host when the mosquito takes a blood meal. It has been suggested that the sporozoites alter the normal salivary functions of the mosquito, thus enhancing the efficiency of their transmission (Beier et al. 1992). Only a small

number penetrate the secretory duct and are injected into the human host. These are then carried in the blood stream and locate and bind to the hepatocytes (liver cells) within a matter of minutes (Nussenzweig and Nussenzweig 1985). Sporozoites can either enter hepatocytes by formation of a parasitophorous vacuole or by disrupting the cell membrane and becoming free in the cytosol (Mota et al. 2001). There is evidence (both *in vitro* and *in vivo*) that sporozoites can transverse several hepatocytes before replicating (Mota et al. 2001). Inside the hepatocytes they undergo the exo-erythrocytic (hepatic schizogony) stage of the life cycle. At this stage, *P. vivax* parasites are able to form hypnozoites that remain dormant in the hepatocytes for a period of time.

Tens of thousands of merozoites are released from each liver schizont and they locate and invade erythrocytes (red blood cells) (Krettli and Miller 2001). The erythrocytic schizogony stage is a form of asexual amplification, and it is this stage that is symptomatic. After an unspecified period of time some of the schizonts will produce the sexual forms of the parasite (female and male gametocytes); these circulate in the blood until a female mosquito ingests them and the cycle begins again.

1.4 The anopheline mosquito life cycle

Adult female *Anopheles* mosquitoes come into contact with humans because they require a blood meal in order to produce eggs; this usually happens at night (Wanji et al. 2003) and can happen either indoors or outdoors depending on the mosquito species (Service 1978). Different *Anopheles* species have differing tastes, with *An. gambiae s.s.* being highly attracted to humans as a source of blood (Costantini et al. 1999, Wanji et al. 2003), whereas some other species, such as *Anopheles arabiensis* Patton prefer to feed on livestock (Bøgh et al. 2001, Rowland et al. 2001, Mahande et al. 2007a). Mosquitoes that feed more on livestock than humans are less efficient malaria vectors (Takken et al. 1999). Female mosquitoes are

attracted to their hosts due to the heat, carbon dioxide and body odours emitted (Takken and Knols 1999, Mukabana et al. 2004). The mosquito injects her proboscis into the human flesh and draws up blood, almost like a hypodermic needle. The blood meal must be sufficiently large to initiate the cascade of hormonal reactions to allow egg production. Therefore, mosquito abdomens are capable of extensive expansion owing to the membranous areas between each set of tergites and sternites. Anopheline females are also capable of excreting the plasma from the blood whilst feeding so that they can concentrate the more nutritious red and white blood cells (Clements 1992).

After the blood meal the females rest to allow digestion to occur, *An. gambiae s.s.* usually rest indoors whereas other mosquito species can rest outdoors (Lines et al. 1986). For successful egg development the ovarian follicles must be in the resting phase when the mosquito takes a blood meal. Egg maturation is dependent on temperature and in tropical Africa usually occurs around three days after the blood meal. At this point she will try and find a suitable water body into which to lay her eggs. Oviposition occurs at night (McCrae 1983) and female mosquitoes can detect competitors (Munga et al. 2006), bacteria (Lindh et al. 2008) and botanical products (Howard et al. Under Review) in the water (**Chapter 4**). After oviposition the female will take another blood meal and so the egg maturation cycle continues. The process of taking a blood meal, egg maturation and oviposition is called the gonotrophic cycle (GC). An anopheline mosquito in tropical Africa usually requires a blood meal every three days. Parasite development in the mosquito (called the parasite extrinsic incubation period (EIP)) can be calculated using the equation:

$$N \text{ (days)} = 111/(T - t_{min})$$

where T is the mean temperature and t_{min} is taken as 16°C (Detinova 1962). Therefore at 27°C the EIP would be 10 days. So, a mosquito must take several blood meals within this period where she will not be infectious (able to spread malaria). Similarly, the mosquito must be relatively old before she can transmit malaria. Female mosquitoes can live for over three weeks (Olayemi and Ande

2009), the conditions most conducive to long female survival are reported to be found in dry, lowland deforested areas (Afrane et al. 2007).

Anopheline eggs are boat-shaped (Figure 1.3), around 1 mm long and do not survive desiccation. Batches of 50-100 eggs can be laid, with larger females laying more eggs (Lyimo and Takken 1993). These eggs are laid singly and have air floats. When the temperature is 25-30ºC the eggs usually hatch after 2-3 days, with the larval head emerging from the anterior pole (Soumare and Ndiaye 2005). Mosquito larvae have four instar stages and have to moult between each instar. The first instar larvae are usually just 1-2 mm long and the final instar larvae can measure up to 10 mm in length. Whilst the larvae are aquatic, there is some evidence that they can survive in moist mud (Miller et al. 2007). Anopheline mosquito larvae lie parallel to the water surface and are surface filter feeders, using brush-like structures to move food towards their mouthparts (Merritt et al. 1992). They breathe atmospheric air through two spiracles located on the eighth segment of their abdomen. The time required for full larval development is dependent on temperature and density (Gimnig et al. 2002), but in tropical areas can take 1-2 weeks. However, not all mosquitoes complete the aquatic phase of the life cycle (Afrane et al. 2007, Olayemi and Ande 2009). Death rates at this stage can be very high, with mortalities as high as 93% recorded for *An. arabiensis* larvae and pupae in Kenya (Service 1977). After completing the fourth larval instar stage the mosquitoes moult into pupae. Mosquito pupae are aquatic and “comma” shaped. They do not feed and *Anopheles* pupae have relatively short and almost conical respiratory trumpets. They spend most of their time at the water surface but will readily dive to the bottom when they detect a shadow on the water or movement. In tropical areas the pupal stage of the lifecycle usually lasts two days.

When the adult mosquito emerges from the pupal skin, the dorsal surface of the cephalothorax splits and the adult mosquito pushes itself out. Once emerged, adult mosquitoes rest for about an hour to let their wings and cuticle harden.

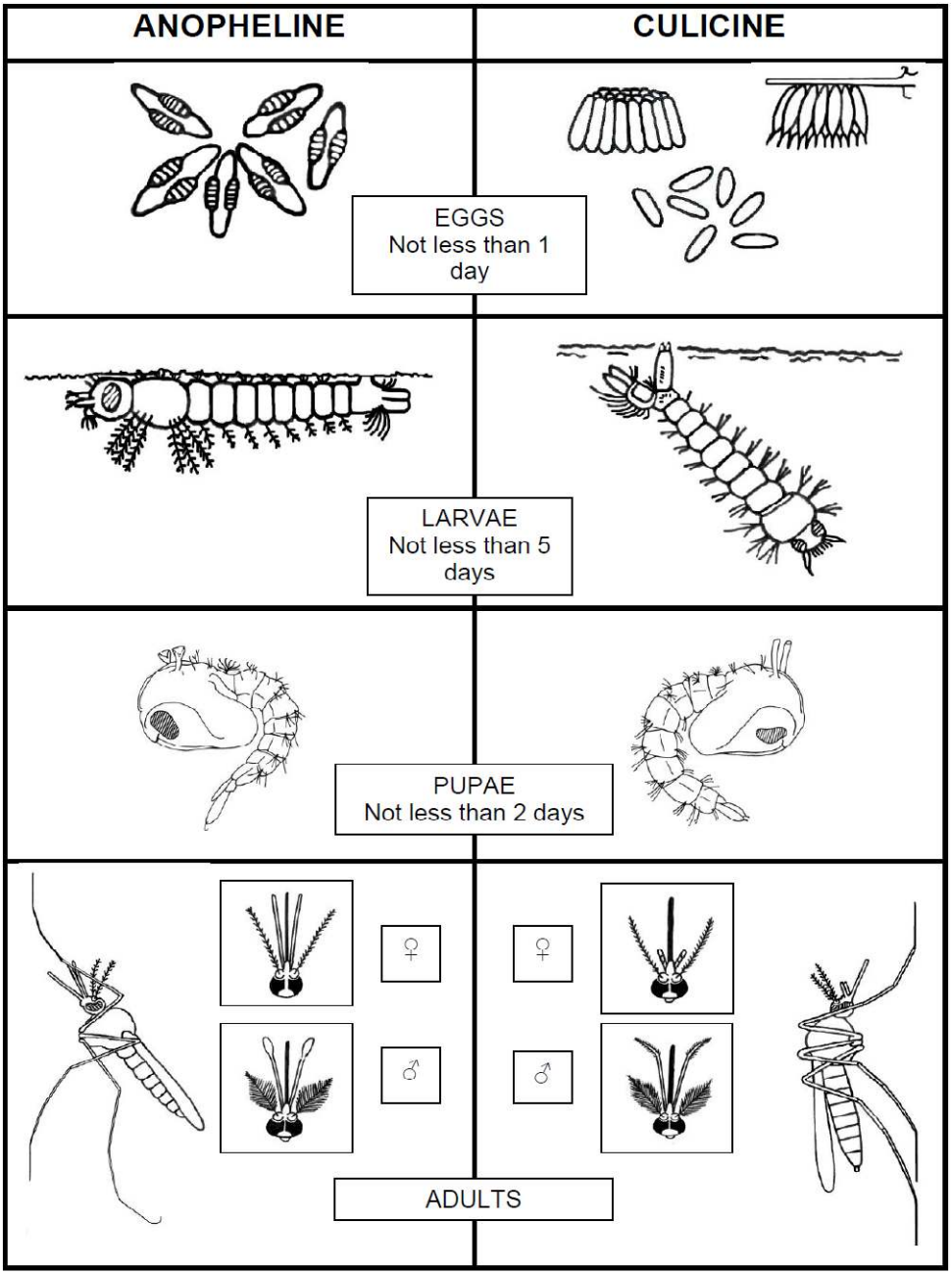

Figure 1.3. Mosquito developmental stages (courtesy of Mrs C. Whitehorn, LSHTM)

They then find sugar/nectar to replenish their energy (Clements 1992) and their next priority is to mate. Males usually form mating swarms at dusk. Females fly into these swarms and are recognised by their different wing beat frequency. Males choose to mate with larger females (Okanda et al. 2002). A pair then drops out of the swarm and mates. Females mate only once in their lifetime, storing sperm in their spermatheca held in place by the mating plug (Rogers et al. 2009).

Whilst adult females will feed on both blood and sugar/nectar, adult male *Anopheles* only feed on sugar/nectar. Adult *Anopheles* usually rest with their body at an angle to the surface with their proboscis and abdomen in a straight line. It is in this way that they can easily be distinguished from the culicine *Culex* and *Aedes* mosquitoes. Other distinguishing features of adult *Anopheles* include the four dark and pale spots along the leading edge of the wing (not seen in the schematic Figure 1.3), and the female palps being the same length as the proboscis (Gillies and Coetzee 1987).

1.5 Anopheline mosquito ecology

The *An. gambiae s.l.* species complex is the most efficient malaria vector system found in Africa (White et al. 1972, Lindsay et al. 1998). This species complex has seven sibling species, the most common and best known are *An. gambiae s.s.*, and *An. arabiensis.* As mentioned in **section 1.4** above, *An. gambiae s.s.* is highly anthropophilic (prefers to feed on humans) and therefore, is an extremely efficient malaria vector. *An. arabiensis* is more zoophilic (prefers to feed on livestock) making this species a less efficient vector. *An. arabiensis* is better adapted to arid areas than *An. gambiae s.s.* (White 1974, Lindsay et al. 1998), however, there is considerable overlap in their ranges (Gillies and Coetzee 1987, Coetzee et al. 2000). *Anopheles funestus* Giles is another species complex (Gillies and Coetzee 1987) that can contribute significantly to malaria transmission in Africa due to its anthropophilic nature. A recent review found that EIR rates were twice as high in

areas where *An. gambiae s.l.* and *An. funestus* were found together when compared to areas where *An. gambiae s.l.* was found alone (Kelly-Hope and McKenzie 2009). Where *An. arabiensis* extends malaria transmission into arid areas, it is thought that *An. funestus* extends malaria transmission in time, by extending the transmission season in rural areas by being more abundant at the end of the rainy season and the start of the dry season (Kelly-Hope and McKenzie 2009).

Whilst adult mosquitoes are free flying and many different species can often be found in the same areas (Minakawa et al. 2002, Kirby et al. 2008), their choice of larval habitats is more species specific. *An. gambiae s.s.* are known to avoid ovipositing in water that contains competitors (Munga et al. 2006) and a study in western Kenyan fishponds found that *An. gambiae s.l.* and *An. funestus* mosquitoes, although using similar types of water bodies to lay their eggs, were tending to avoid ovipositing in the same fishpond as the other species (Howard and Omlin 2008) (**Chapter 5**). This said, overlap of different species in larval breeding sites can often be found (Gimnig et al. 2001, Howard et al. 2007) (**Chapter 6**) and even within species the larval habitats can be varied.

In general, anopheline larvae can be found in non-organically polluted, usually natural, water bodies. The biotic and abiotic factors necessary for mosquitoes to colonise water bodies have been studied, however, different factors have proven more important in different settings. One study found that typical breeding sites for *An. gambiae s.s.* included small non-permanent habitats with algae and little or no vegetation (Gimnig et al. 2001). Another study, although agreeing that vegetation was not important, found that the abundance of *An. gambiae s.s.* larvae was not significantly associated with a number of other variables including algae (Minakawa et al. 1999). In Kenya *An. arabiensis* were associated with small non-permanent habitats with little or no vegetation (Gimnig et al. 2001). However, *An. arabiensis* are regularly found breeding in rice fields (large semi-permanent habitats), where there is vegetation. *An. arabiensis* breeding in rice fields have been associated

with a number of variables including low turbidity (clear water), dissolved oxygen and water depth (Mwangangi et al. 2007), while in wells in Senegal, important characteristics appear to be relatively shallow water (<0.5 m) that is warm and clear (Robert et al. 1998). *An. funestus* larvae have been associated with relatively large semi-permanent habitats with vegetation and algae (Gimnig et al. 2001), although another study found no significant relationship with vegetation (Howard and Omlin 2008) (**Chapter 5**).

An. gambiae s.l. is increasingly being reported to colonise man-made water bodies. A study in western Kenya found that 87.5% of anopheline positive larval habitats were man-made, and many more *Anopheles* were found in these brick making sites than in the nearby swamps (Carlson et al. 2004). Other man-made habitats found to contain anopheline larvae include fishponds (Howard et al. 2007, Howard and Omlin 2008) (**Chapters 5 & 6**), swimming pools (Impoinvil et al. 2008) and burrow pits (Mutuku et al. 2006b). Agricultural land use is also associated with significantly more *An. gambiae s.l.* habitats when compared to land used for non-agricultural purposes (Mutuku et al. 2009). Mosquito distribution is also dependent on the availability of blood hosts for the adults, and *An. gambiae s.s.* larvae are more commonly found closer to human habitations than to animal sheds (Minakawa et al. 2002). Clearly, human activity is greatly influencing the distribution of anopheline, and especially *An. gambiae s.l.,* larvae.

1.6 The concept behind mosquito/vector control

The vectorial capacity equation (MacDonald 1957) is used to estimate the capacity of a mosquito population to transmit malaria in terms of the potential number of secondary inoculations originating per day from an infective person. This equation is expressed as:

$$C = ma^2p^n / -\ln p$$

where m is the number of vectors per host, a is the number of blood meals taken on humans per vector per day, p is the daily survival rate for the vectors and n is the rate of parasite development in the mosquitoes (the EIP). Of all these parameters, the most important are considered to be the daily human biting rate and the daily vector survival. This is because the daily human biting rate is squared in the equation and the daily vector survival is raised to the power of the parasite development rate. Therefore, even small reductions in these two parameters can have large effects on the vectorial capacity of the local mosquito population (Garrett-Jones 1964). Once the longevity of the mosquito is decreased below the minimum time for the EIP of the malaria parasite inside the mosquito, then malaria transmission can be interrupted.

The ways to tackle these two parameters are to reduce the human-vector contact (by screening, bednets, and repellents) and to kill the mosquitoes. ITNs are able to do both these things and this is why their use is the mainstay of many major malaria control programmes. Because some insecticides can be repellent, IRS can also be used to reduce these two parameters, although IRS reduces the human-vector contact to a lesser extent (more on these methods in **sections 1.7.1 & 1.7.2**). Although the main focus of vector control campaigns is the adult female mosquitoes, the effectiveness of adult control can be reduced because adult mosquitoes are highly mobile and can choose to avoid coming into contact with the control methods (Killeen et al. 2002). Also behavioural changes resulting from selective pressure, such as earlier feeding in some populations, mean that they do not contact the insecticides on ITNs because they feed before the people have gone to bed (Pates and Curtis 2005).

Whilst reducing the number of mosquitoes using larval control can also be important, this causes less of a reduction in the vectorial capacity equation than reducing the human biting rate and daily survival rate of the adult mosquitoes. Larval control in Africa has also been considered to be too labour intensive because the main malaria vector, *An. gambiae s.l.*, can be found in a variety of

water bodies as small as individual cow hoof prints (Minakawa et al. 1999). Another problem with larval control is that adult female mosquitoes do not oviposit indiscriminately, avoiding competitors (Munga et al. 2006) and predators (Angelon and Petranka 2002, Blaustein et al. 2005). For these and other reasons larval control has not been used on a large scale to tackle malaria transmission in Africa. However, recent work has indicated that targeted larval control campaigns could be effective. Along with the variety of other breeding habitats, African anopheline mosquitoes tend to breed in man-made pits of water (Carlson et al. 2004, Fillinger et al. 2004, Howard and Omlin 2008) (**Chapter 5**). These pits are thought to be much more productive than the numerous smaller water bodies because it has been found that *An. gambiae s.l.* pupal occurrence is positively correlated with both habitat size (Minakawa et al. 2005) and habitat stability (Mutuku et al. 2006b). Another positive factor for larval control is that mosquito larvae cannot disperse from the water body into which the female mosquitoes laid the eggs.

It will never be possible to treat every single anopheline mosquito habitat with a larval control tool, however, targeting these relatively large, man-made and productive habitats (Mutuku et al. 2006b) could help reduce the density of mosquitoes in a given area (Killeen et al. 2002). A study in western Kenya found that more than 90% of adult anopheline mosquitoes caught inside houses were caught within 300 metres of the nearest larval habitats, and that *An. gambiae s.s.* were more likely to be found in water bodies close to human habitations than close to animal sheds (Minakawa et al. 2002). The location of water bodies close to human habitations are usually well known, especially if they are man-made. Even if the location of relatively large water bodies are not known, new tools such as geographical information systems exist to identify such habitats (Mushinzimana et al. 2006) and larval control trials in Africa have already proved successful (Fillinger et al. 2003, Geissbuhler et al. 2009). These factors make the inclusion of larval control into malaria control programmes a viable option.

1.7 Current vector control methods used

1.7.1 Insecticide-treated nets (ITN)

Sir Ronald Ross, the man who finally completed the malaria-mosquito-man puzzle, slept under a bednet in the late 19th century as protection from nocturnal mosquito bites (Gibson 1998). Bednets are still used to protect against mosquitoes, however, they are now treated with pyrethroid insecticides giving them a dual function. Firstly their physical barrier, enhanced by the irritant properties of the insecticide, reduces blood feeding from humans. The second function is the reduction of the mosquito population density (Bayoh et al. 2010), average mosquito age (Magesa et al. 1991, Vulule et al. 1996) and in some areas species composition (Lindblade et al. 2006, Bayoh et al. 2010) due to the insecticide treatment. Disease reduction is achieved through personal protection against biting insects and reduction in the transmission intensity. The transmission intensity decreases because the number of infectious mosquitoes is reduced due to their inability to feed from gametocyte positive people, and a reduced number of mosquitoes that live long enough for the malaria parasite to complete its (EIP) lifecycle. ITNs are seen as a targeted use of insecticides because the sleeping person attracts the mosquitoes to the insecticide.

Treated nets are efficient at reducing malaria mortality and morbidity in high and low transmission areas (Abdulla et al. 2001, Maxwell et al. 2002, ter Kuile et al. 2003). As well as the personal protection afforded by ITNs, if a large proportion of the community uses ITNs then a decrease of the vector population can be expected (Curtis et al. 2003). This ‘mass effect’ has been seen in areas such as Tanzania (Magesa et al. 1991) but was not seen in The Gambia despite high coverage, thought to be because the intervention and control village mosquitoes shared breeding sites (Lindsay et al. 1993b).

ITNs have their limitations. Net treatment is important, Lines et al. (1987) showed that an untreated net can be more dangerous than no net due to the deflection of biting to another person in the same room. In Tanzania a recent study found that

most nets were not treated with insecticide, partly due to misconceptions about the effect of the insecticide on human health (Matovu et al. 2009). ITNs should be treated at least every 6-12 months or after every 3 washes (Gonzalez et al. 2002). In many areas this is not carried out (Matovu et al. 2009) and this lack of re-treatment is not always linked to cost. In a voucher scheme where 80% of the cost was subsidised only 1% of vouchers were used (Armstrong Schellenberg et al. 2002).

Long-lasting ITNs have been developed to counter some of the limitations of traditional ITNs. Long-lasting nets have been manufactured in such a way that they do not require further insecticide treatment and remain effective under field conditions for around 5 years. Although many have been developed, only a handful are recommended for use by WHO (World Health Organisation 2009). Long-lasting ITNs have been shown to be more cost effective than traditional ITNs in a variety of countries where both types of nets were either given out or highly subsidised (Yukich et al. 2008). However, long-lasting ITNs are more expensive than traditional ITNs so in areas where people have to buy nets, they are not reaching the resource-poor rural communities (Matovu et al. 2009) that most need them. To try to prevent this imbalance, WHO released a press statement in 2007 saying that "WHO recommends that insecticidal nets be long-lasting, and distributed either free or highly subsidized and used by all community members" (World Health Organisation 2007).

Many countries in Africa and other areas have followed the WHO recommendation and now have a policy of providing treated nets to all age groups that are at risk of malaria, whereas previously just pregnant women and children were targeted (World Health Organisation 2009). However, despite the WHO/WHA/MDG goal that 80% of people at risk of malaria should be sleeping under a treated net by 2010, WHO estimates that in Africa only Mali and São Tomé and Principle have so far achieved this target (World Health Organisation 2009). The 2009 World Malaria Report estimates that in 2008, on average just 31% of African households owned a

treated net and 24% of children under the age of 5 were using them (World Health Organisation 2009).

Sixty-eight countries are currently distributing treated nets for free (World Health Organisation 2009). However, even handing out ITNs cannot guarantee wide scale coverage because there is some evidence that within a few years of ITN distribution these nets are not being used. In Sierra Leone 2-3 years after a mass distribution campaign household ITN ownership had reduced by 37%; in Togo the decline was 13% (World Health Organisation 2009). In Ethiopia one study found that although ITN ownership was 91%, only 65% had used the net the previous night (Baume et al. 2009). There are several reasons why people do not use treated nets. Firstly, bednet use can be seasonal. When mosquito populations decrease people tend to stop using bednets, however, the mosquitoes left are usually old and more likely to be infectious (Lines et al. 1991). Bednets can also be hot and stuffy to sleep under and some houses are not large enough for their use (Majori et al. 1987). Also, people can (mis)use bednets for a variety of other activities such as for fishing nets (Minakawa et al. 2008) and even wedding dresses (Odeke 2002). Furthermore, even when being used, ITNs can have reduced efficacy because nets can get holes in them (Tami et al. 2004), negating the protective value of the physical barrier (Irish et al. 2008). Although less so for long-lasting nets, insecticides can wear off under field conditions and a study looking at long-lasting nets used in the field for 7 years found that they were no longer able to protect against blood feeding (Malima et al. 2008). Growing insecticide resistance is also threatening the usefulness of treated nets in some areas (N'Guessan et al. 2007) (more on this in **section 1.9.1**). In spite of these problems, even the poorest families in malaria endemic areas consider treated nets a priority (Armstrong Schellenberg et al. 2002).

1.7.2 Indoor residual spraying (IRS)

When mosquitoes enter a house to blood feed they tend to rest on the walls or ceiling immediately before and after feeding. The aim of IRS is to leave long-lasting residual insecticide on the ceiling and walls of houses so as to increase the risk of a mosquito being killed each time it enters the house. This reduces the chance of an infected mosquito living long enough for the malaria sporozoites to mature. When the WHO initiated the Global Malaria Eradication Campaign in 1955, IRS with dichlorodiphenyltrichloroethane (DDT) was the mainstay of this campaign. Despite malaria being successfully eradicated from areas including North America and Europe, many places in Africa were not targeted due to the lack of health infrastructure and high intensity transmission; just three African countries joined the campaign. Less than 20 years later, the emphasis was officially shifted from eradication to vector control (Hemingway and Ranson 2000). The main reasons for this were the occurrence of DDT-resistant mosquitoes (Curtis and Lines 2000), the cost of the campaign (Litsios 2000) and growing public dissatisfaction with DDT (see **section 1.9.2**).

Currently IRS is the primary vector control intervention in 45 countries and in 2008, it protected 59 million people (World Health Organisation 2009). There are 12 insecticides that can be used for IRS (World Health Organisation 2009), however, these only encompass three different toxic modes of action (van den Berg 2009). Lambdacyhalothrin has been used in Angola (Somandjinga et al. 2009) and Uganda (Bukirwa et al. 2009) but insecticide resistance is threatening the efficacy of IRS with lambdacyhalothrin in Benin (N'Guessan et al. 2007). Carbamate is used in Mozambique (Yukich et al. 2005) and other insecticides such as chlorfenapyr are being tested for their potential use (N'Guessan et al. 2009). Despite some controversy over the accumulation of DDT in the environment (more on this in **section 1.9.2**) and the effect on non-target organisms, DDT is still used for IRS in South Africa, Mozambique (Yukich et al. 2005) and other countries (Sadasivaiah et al. 2007, van den Berg 2009) to great effect.

IRS had a huge impact on mosquito populations and malaria transmission during the eradication campaign of the 1950-60s. This is exemplified by Sri Lanka which saw malaria cases fall from 2.8 million during the 1934/35 epidemic to just 17 cases in 1963 (Curtis and Lines 2000). Of the great entomological achievements, IRS in South Africa managed to drive the main malaria vector *An. funestus* back to the Mozambique border (Hargreaves et al. 2000). Even now IRS is able to control malaria, especially in areas of unstable malaria transmission (Pluess et al. 2010). In Uganda, IRS with the pyrethroid lambdacyhalothrin was able to significantly reduce both the numbers of people diagnosed with clinical malaria, and the number of blood slides found to contain malaria parasites in the first four months after spraying (Bukirwa et al. 2009). Unfortunately, this study found that the beneficial effects of the spraying wore off, and a year after spraying the proportion of positive blood smears was not significantly different from pre-spraying levels (Bukirwa et al. 2009). It is known that the residual effects of the insecticides wear off and for this reason IRS is usually carried out every 6 months or so. This can impose logistical problems as an IRS campaign can be both expensive and logistically demanding because IRS should be carried out using specific equipment used by trained people. Successful IRS campaigns require wide-scale coverage to be effective; if a high percentage of the houses have been sprayed then very few mosquitoes will avoid a lethal dose. In addition, IRS has benefits other than affecting mosquito populations. House spraying in Zimbabwe was able to reduce the odds of a failure of chloroquine treatment fourfold but this effect was not seen after spraying was stopped (Mharakurwa et al. 2004), and other household insect pests are killed.

When pyrethroid spraying was directly compared to ITNs there was no significant difference between the two methods with respect to vector control, but the ITNs used just 1/6 of the insecticide that IRS used, and ITNs were greatly preferred by the villagers (Curtis et al. 1998). For this and for the logistical reasons, IRS is not as cost effective as the use of ITNs (Yukich et al. 2008), however, they have slightly different functions; ITNs are used to protect specific people whereas IRS is

used to protect communities and respond to epidemics.

1.7.3 *Bacillus thuringiensis* var. *israelensis* (Bti)

As discussed in **section 1.6** above, there is an argument for targeting vector control at mosquito larvae as well as at the adults. Examples of effective larval control tools that are currently not in widespread use will be reviewed in detail in **Chapter 2**.

One larval control tool that has received much attention in recent times is Bti, a Gram positive bacterium that can be used to target specifically the larvae of mosquitoes and some flies (e.g. *Simulium* the vector of the river blindness parasite). The mode of action of Bti requires oral ingestion by the mosquito larvae. Bti bacteria produce δ-endotoxins in parallel with spore formation during the stationary phase of the cell cycle. These toxins form crystalline inclusion bodies around the spores that are produced during sporulation. The crystals are toxic to mosquito larvae because the alkaline nature of the mosquito digestive tract dissolves the δ-endotoxin crystals. Mosquito enzymes then cleave the pro-toxins to create toxins. These activated toxins bind to the cell membranes of the mosquito's gut, forming pores and disrupting cellular osmotic balance leading to cell rupture and death. Mosquito pupae do not feed and as such are not susceptible to Bti. Due to the specificity of Bti, non-target organisms within water bodies are not adversely affected, so Bti can be used in conjunction with biological control agents in an integrated vector management (IVM) strategy (see **section 1.7.4** below) (Hurst et al. 2007, Lacey 2007).

Although Bti has been shown to be highly effective at reducing mosquito numbers in the field (Fillinger et al. 2003, Kahindi et al. 2008) and can reduce the risk of malaria infection (Geissbuhler et al. 2009) it has poor residual activity under field conditions. In western Kenya successful recolonisation of treated areas was evident 2-3 days after treatment (Fillinger et al. 2003) and in The Gambia

standardized field trials showed that weekly retreatment was necessary to achieve a constant suppression of mosquito larval development (Majambere et al. 2007).

Furthermore, the persistence and efficacy of Bti in a water body depends on several bioenvironmental factors such as the levels of organic pollution, the presence of vegetation and formulation used (Mittal 2003). Formulation is important because *Anopheles* larvae feed at the surface, formulations that delay or prevent Bti settling at the bottom of the water body can be effective for longer periods. Bti is also inactivated by ultra violet (UV) light which reduces the longevity of action in the field. For these reasons frequent repeat applications are required (Gunasekaran et al. 2004, Majambere et al. 2007).

Weekly retreatments of a product that is already expensive to produce raises the cost of effective larval mosquito control, but Bti has previously been produced in a cheap and sustainable way in a resource-poor area of Peru. Whole coconuts were inoculated with 7.8×10^5 spores of Bti and after 48-96 hours the coconuts were thrown into nearby ponds. Bti persisted in these ponds for 15-25 days and was able to successfully control *Anopheles* larvae (Ventosilla et al. 1990). Despite this encouraging work 20 years ago, there appears to have been no trials of this method in Africa, maybe because it is difficult to inject a coconut! Waste products that are found in resource-poor tropical countries can be used to grow Bti (Prabakaran et al. 2008) and it is hoped that this will bring the cost of this mosquito control product down. Bti is commercially available from many companies and its specificity to mosquitoes and other flies means that it cannot be sold on the black market as an agricultural insecticide to target agricultural pests.

1.7.4 Integrated vector management (IVM)

It is widely accepted that malaria control will only be successful if an integrated approach is taken, focussing on both vector control and chemotherapy (treating the human parasitic infection with ACTs) (World Health Organisation 2009). In addition,

it is becoming increasingly apparent that malaria transmission cannot be stopped by one single vector control method. IVM "integrates all available resources to achieve a maximum impact on vector borne disease" and was formally adopted by WHO in 2004 (World Health Organisation 2004b). It is thought that IVM will improve efficiency, cost-effectiveness and the sustainability of disease control. Similarly, because many different techniques are to be used in conjunction, the selection pressure of drug or insecticide resistance is reduced. More in-depth aspects and examples of IVM are discussed in **Chapter 2**.

Almost one hundred years ago the mainstay of mosquito control was environmental management (Utzinger et al. 2001), house modification (Lindsay et al. 2002) and, where appropriate, biological control with organisms such as fish (Austen 1919). Many of these vector control methods were used successfully before being discarded in favour of DDT and the synthetic pyrethroids (World Health Organisation 1982). These "old" mosquito control methods are as applicable today as they were 100 years ago, and in many cases the technologies involved have not changed. They have all proved to be effective (for more on this see **Chapter 2**) and could complement the use of ITNs and IRS in IVM schemes where locally appropriate. In addition, these methods do not use insecticides and so should not compound the problems discussed in **section 1.9** below.

1.8 New malaria control tools in development

1.8.1 Entomopathogenic fungi

The entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* can be used to target mosquito adults (Scholte et al. 2003a, Achonduh and Tondje 2008) and larvae (Clark et al. 1968, Scholte et al. 2004b). The conidia of these fungi, once germinated, directly penetrate the adult mosquito cuticle then produce compounds that can cause insect death (Gillespie and Clayton 1989). For

mosquito larvae the fungal conidia are either ingested through the mouth or enter the siphon. Here they can cause a physical blockage by vegetative growth of the fungi, and the release of midgut toxins causes damage and death to the larvae (Bukhari et al. 2010). Very little work using these fungi against mosquito larvae has been carried out in the last 20 years. The only exception being one recently published article by Bukhari et al. (2010) that showed no differences between the susceptibility of *An. gambiae s.s.* and *Anopheles stephensi* Liston to *M. anisopliae* and *B. bassiana,* however, *B. bassiana* was found to be less effective at killing the mosquito larvae when compared to *M. anisopliae* (Bukhari et al. 2010).

Work using these fungi against mosquito adults is at a more advanced stage. Both *M. anisopliae* and *B. bassiana* have been shown to significantly reduce the longevity of adult mosquitoes using a variety of different experimental procedures in the laboratory (Scholte et al. 2003b, Farenhorst et al. 2008, Mnyone et al. 2009b) and field (Scholte et al. 2005, Lwetoijera et al. 2010). As well as causing mosquito mortality, interesting pre-lethal effects have also been reported. After exposure to *M. anisopliae*, fewer *An. gambiae s.s.* took a subsequent blood meal when compared to the control group (Scholte et al. 2006). Fungus-exposed mosquitoes also laid less eggs per GC resulting in a lower life time fecundity (Scholte et al. 2006). Similarly, in *An. stephensi* mosquitoes infected with *B. bassiana*, the fungus interfered with the ability of the mosquito to take a blood meal (Blanford et al. 2005). In this same study it was shown that *B. bassiana* inhibited the development of malaria parasites within the mosquito, reducing the sporozoite rate. It was hypothesised that fungal exposure could reduce the risk of malaria transmission by a factor of about 80 due to the reduction in sporozoite rate and increased mortality of the fungal-exposed mosquitoes (Blanford et al. 2005).

Questions still remain about fungal longevity and viability under tropical conditions. In Tanzania, *M. anisopliae* in suspension did not lose viability whereas when the fungus was impregnated onto black cotton cloths and exposed to the ambient heat and humidity, the viability had reduced to 63% three weeks after application

(Scholte et al. 2005). This inability of entomopathogenic fungi to withstand tropical temperatures has also been found in several laboratory studies (Rangel et al. 2005, Lekimme et al. 2008, Darbro and Thomas 2009). Although the use of entomopathogenic fungi in the laboratory has produced some encouraging results, further work must be carried out in the field before this malaria control tool can become operational.

Chapters 7 & 8 of this thesis examine the use of entomopathogenic fungi against insecticide-resistant mosquitoes both in the laboratory and under field conditions in Benin, West Africa.

1.8.2 Vaccines

A 1973 paper described how irradiated malaria sporozoites were able to elicit an immune response in humans by causing an antibody response to the circumsporozoite protein (Clyde et al. 1973). Despite this early success, the development of a malaria vaccine has been hampered mainly because, unlike viruses that have relatively simple protein shells, malaria parasites have several different life stages, all with different proteins on the parasite surface. Protistan parasites are also much larger than viruses, making it more difficult for the immune system to overcome them. Because of the different life stages of the malaria parasite, many different antigens were targeted for vaccine research. These included targeting the pre-erythrocytic/asymptomatic life stage, the erythrocytic/symptomatic stage and sexual stage inside the mosquito. Details of all the different types of malaria vaccines developed can be found in a recent review by Chattopadhyay and Kumar (2009). Currently the RTS,S vaccine is the most advanced malaria vaccine candidate and is in phase III trials in Africa (Ballou 2009). This vaccine is aimed at protecting small children and targets the circumsporozoite protein of the sporozoite (the life stage of the parasite injected into humans by the mosquito), and so this vaccine aims to attack the malaria

parasites in the pre-symptomatic stage.

Vast sums of money have been ploughed into research looking for an effective malaria vaccine. The problem seems to be that the more technical the question, the longer it takes to solve and the more money it costs. Vaccine research has been ongoing for more than 35 years and yet the best estimates are that we are still years away from having a usable vaccine (Chattopadhyay and Kumar 2009, Greenwood and Targett 2009). Despite this, undoubtedly, a vaccine for malaria would be a great tool to have in the control arsenal, especially if the goals of global malaria eradication are to be achieved (Greenwood and Targett 2009).

1.9 What are the problems with the current vector control methods?

Although the present vector control methods are showing great successes, as discussed above, they are not infallible. Along with the specific problems mentioned in **section 1.7**, there are other reasons why there is growing focus on the more natural and low-tech control methods.

1.9.1 Insecticide resistance

Insecticide resistance refers to the ability of an insect to tolerate doses of an insecticide that would prove lethal to the majority of individuals in a normal population of the same species. Inheritable resistance traits develop by selective pressure exerted on a mosquito population. As well as reducing the capacity to repel and kill mosquitoes, there is also evidence that insecticides can select for certain behaviourally resistant traits such as earlier mosquito feeding times and earlier exiting from houses with treated nets (Mathenge et al. 2001, Pates and Curtis 2005). The short generation time and prolific progeny characteristic of the

mosquito lifecycle is well suited for quick development of resistance, and over 50 species of *Anopheles* are reported to be resistant to insecticides (Hemingway and Ranson 2000). Resistance usually arises independently in each species and may not be found in all vectors in a malarious area.

Because pyrethroids are the only insecticide class that has World Health Organisation Pesticides Evaluation Scheme (WHOPES) approval for use on ITNs, pyrethroid resistance in an area can seriously hamper vector control activities (N'Guessan et al. 2007). There are several known types of pyrethroid resistance. The knockdown resistance (*kdr*) mechanism, which causes site insensitivity (Enayati et al. 2003), was first reported in West African mosquitoes in the early 1990s (Martinez-Torres et al. 1998). Resistance can also be caused by increased detoxification of the insecticide which is caused by elevated glutathione-S-transferase (Brengues et al. 2003) or mixed function oxidase activity (Vulule et al. 1999). Unfortunately for vector control schemes, resistance to one pyrethroid can confer cross-resistance to the other pyrethroids and a variety of other insecticides used for IRS including DDT (Chandre et al. 1999b, Brooke et al. 2001, Brengues et al. 2003, Enayati et al. 2003).

Part of the problem of adult mosquito insecticide resistance is the presence of sub-lethal doses of agricultural insecticides in the aquatic larval habitats. In this way it is thought that insecticide resistance in mosquitoes was mainly selected for by agricultural insecticides (Lines 1988, Diabate et al. 2002, Akogbeto et al. 2005, Corbel et al. 2007, Yadouleton et al. 2009). Domestic use of volatile pyrethroids can also select for pyrethroid resistance (Kang et al. 1995, Diabate et al. 2002). Extensive use of IRS during the eradication campaign in the 1950-60s caused DDT resistance to occur (Curtis and Lines 2000) and there is also evidence that ITNs themselves select for resistance. Some evidence suggests they select for the *kdr* allele (Fanello et al. 1999, Kolaczinski et al. 2000). However, ITNs are not thought to select for pyrethroid resistance when the *kdr* mutation is rare and heterozygous (Kang et al. 1995, Corbel et al. 2004). There is also evidence that ITN use selects

for higher levels of oxidases and esterases in mosquitoes (Vulule et al. 1999).

The problem of insecticide resistance is increasing, however, despite this widespread and almost global problem (Chandre et al. 1999b, Vulule et al. 1999, Hargreaves et al. 2000, Baleta 2009), in some instances insecticides can still be effective against insecticide-resistant mosquitoes. In Côte d'Ivoire, the *kdr* allelic frequency almost doubled to 92.5% after just one year of ITN use, however, child malaria morbidity halved (Chandre et al. 1999a). Furthermore, when 80% of the population in an area of pyrethroid resistance were protected with ITNs, the ITNs were able to significantly reduce the prevalence of malaria in children, with a 56% protective efficacy against malaria (Henry et al. 2005).

This ongoing protective effect in resistant areas is probably because resistance reduces the irritability of the pyrethroids allowing the mosquito longer net-contact time. Thus, even though for resistant mosquitoes the lethal dose may be higher, the increased net-contact means this dose is likely to be reached. Being less irritated, resistant mosquitoes are also more likely to find a way into the net and get trapped inside. However, this reduced irritancy does mean that treated ITNs in some areas are no longer able to protect against blood feeding (Guillet et al. 2001, Asidi et al. 2004, N'Guessan et al. 2007).

1.9.2 Insecticide accumulation in the environment

Whilst in the past little regard was paid to the environmental cost of disease control, this is increasingly becoming a priority. The 2001 Stockholm Convention on Persistent Organic Pollutants succeeded in banning several pesticides but DDT won a reprieve (Sadasivaiah et al. 2007); the use of DDT for specific activities like malaria control was allowed but subject to very strict regulations. The reason why these pesticides were banned, and the use of DDT was restricted, was because chemicals can build up in the oceans, air, soil, food chain, fresh water supplies (Mansour 2009, Ogata et al. 2009, van den Berg 2009), and adversely affect non-

target organisms (including humans) (Eskenazi et al. 2009). Further, DDT can spread to areas where it has not been used because it is very volatile in the warm, tropical areas where it is used. It then gets transported in the air and is deposited in high altitude areas where, being colder, it is less volatile and more likely to remain (van den Berg 2009). In short, chemical insecticides like DDT can cause environmental devastation if their use is left unchecked. The environmental accumulation and correct disposal of pesticides has been affected by poor or inappropriate storage facilities, a lack of trained staff and lack of adequate government monitoring (Haylamichael and Dalvie 2009). The fact that the agricultural sector uses the same pesticides as disease control does has further accentuated these problems.

DDT, the insecticide used in the 1950s eradication campaign and still in use today, can accumulate in human breast milk (Azeredo et al. 2008, Sereda et al. 2009). In South Africa it is thought that this is directly due to contact with DDT used for IRS for malaria control. A recent study examined whether DDT in breast milk was higher in women exposed to IRS compared to women who were not exposed to IRS, but who ate the same food. It was found that the IRS-exposed women had much higher levels of DDT in their breast milk when compared to non-exposed women. DDT levels in the food, bovine milk and drinking water sources were not high enough to be considered as additional causes of this difference (Sereda et al. 2009). This accumulation in human tissues can cause problems; DDT in breast milk in Brazil resulted in 8.7% of children examined being exposed to higher daily levels of DDT than WHO recommends (Azeredo et al. 2008). DDT has also been linked to breast cancer, diabetes, impaired neurodevelopment in children and even spontaneous abortion (Eskenazi et al. 2009, van den Berg 2009). Given this evidence, the fact that DDT is still allowed for use for malaria control is testament to its cost-effective mosquito killing ability, and to the fact that the tool cupboard for malaria control is not as full as we would like. Although pyrethroid insecticides are not as harmful as DDT (they are, after all, the only insecticide class permitted for use on ITNs), they can also accumulate in the environment (Yanez et al. 2002) and

in human breast milk (Sereda et al. 2009).

1.10 Why should we be focussing on different malaria control techniques?

As can be seen in the preceding sections, there are several problems with current malaria control techniques. Although increasing, ITN coverage still falls well short of the WHA/WHO/MDG goals in many countries (World Health Organisation 2009). In areas where ITNs are on the ground, they are not being used properly for a variety of reasons. Some cultures find them unacceptable, or their houses are not big enough (Majori et al. 1987), some ITNs are being misused (Minakawa et al. 2008), or not being used due to a misconception about the amount of mosquitoes around (Baume et al. 2009). In some areas political instability means people do not sleep in a bed in their own home (Medlock et al. 2007), and so would have little chance to use an ITN. IRS is logistically demanding and can be expensive (Yukich et al. 2008), and the insecticides used can accumulate in human tissue (Sereda et al. 2009). The insecticides used on ITNs are safer for humans but mosquitoes are becoming resistant (Hargreaves et al. 2000). Bti can be effective but is expensive and requires frequent repeat applications (Majambere et al. 2007).

Furthermore, rural African communities are in general poorer (Matovu et al. 2009), less healthy (Hay et al. 2005), with less access to health facilities (Noor et al. 2003), and more contact with malaria vectors (Kirby et al. 2008) leading to a higher malaria burden (Kelly-Hope and McKenzie 2009) when compared to urban communities. Rural communities are the most in need but in many instances they are the hardest to reach. In resource-poor rural areas of sub-Saharan Africa there are many logistical difficulties facing organisations trying to improve ITN coverage or involved in IRS campaigns. Many of the roads are poor and during the wet season may become impossible to use. The lack of decent roads impedes efficient delivery of mosquito control tools. Another problem of the lack of infrastructure is

that due to the lack of piped water sources for rural African communities, many need to keep water sources for domestic use; these water bodies are sources of malaria vectors (Mutuku et al. 2006a, Imbahale et al. 2010).

So what are the alternatives to achieve effective malaria control, or even elimination? It seems that many people are focussing on entomopathogenic fungi (**section 1.8.1**) and the malaria vaccine (**section 1.8.2**). Undoubtedly, when these tools become operational they will be great additions to the control arsenal, especially if the goals of global malaria eradication are to be achieved (Greenwood and Targett 2009). However, at present neither can be used for large-scale malaria control.

So what now? What can we use whilst waiting for ITN/IRS/IVM coverage to increase and for the vaccine to be successfully developed and rolled out? Given that many communities in Africa are not presently being protected by the available tools (ITN, IRS, Bti) and given the unavailability of advanced tools (vaccine, transgenic refractory mosquitoes, sterile male mosquitoes) for malaria control in the immediate future, much work is focussed on developing and rediscovering malaria control tools that are affordable to the poorest people and available now. It is important to investigate ways in which the local communities can control mosquitoes using locally available materials because involving local communities should allow for more successful and sustainable malaria control (Mukabana et al. 2006). There are many vector control techniques that can be relatively easily and cheaply implemented by the people most at risk from malaria. These include house modification (Kirby et al. 2009), environmental management (Keiser et al. 2005), biological control (Mohamed 2003), the use of plants as larvicides (Kihampa et al. 2009) and repellents (Waka et al. 2004), and zooprophylaxis (Mahande et al. 2007b). Each of these methods will be discussed in greater detail in **Chapter 2**.

1.11 Problem definition, research objective and thesis outline

Despite effective tools being available for successful malaria control, certain logistical issues are impeding the roll out of IVM strategies involving ITNs and IRS. Even without these logistical issues, ITNs and IRS are not infallible and problems such as insecticide resistance are threatening to undermine their use. Before the advent of synthetic insecticides, malaria was controlled with a variety of natural control methods. Although these methods were rarely as effective as the current methods that utilise insecticides, they were still able to impact on mosquito populations.

There is a disproportionate malaria burden in Africa. Not only are rural communities more at risk, they are also less equipped to deal with this burden. In general they have less protection (ITNs) less access to health facilities and less money. There are several low-tech malaria control techniques that lend themselves for use in resource-poor rural areas. Many of them utilise free and readily available natural resources (many plants), can self replicate (larvivorous fish) or that may already be part of the household (animals for zooprophylaxis). While these methods may not be better than the current insecticide-based tools in use, they are better than nothing, and could be utilised until the vaccine has been licensed and ITN and IRS coverage is 100%. Furthermore, the focus of IVM is to use many tools simultaneously.

Focus is turning to natural control tools partly as a result of environmental accumulation of insecticides, and partly due to insecticide resistance. In addition, biodegradable substances are being investigated because more emphasis is being placed on keeping ecosystems stable and avoiding environmental pollution. This is especially important because insecticides can affect mosquito predators more adversely than the mosquitoes themselves, because mosquitoes tend to colonise areas faster than their natural enemies (Service 1978). In addition, the WHA resolution 50.13 calls for the development and adoption of viable alternative

methods of controlling vector-borne diseases to thereby reduce the reliance on insecticides (World Health Organisation 2004b).

Research into readily available, natural, and potentially sustainable malaria control tools is required so that resource-poor rural communities can take ownership of the problem of malaria control. In addition, with WHO recommending the use of IVM for malaria control, the long proven techniques of biological control should be given more focus so that they can slot into the IVM arsenal.

The **research objective** was to examine the feasibility and effectiveness of the use of three natural products (flora, fish and fungi) for malaria vector control. These products are introduced in **section 1.8.1** above (fungi), **section 2.3.2** (fish) and **section 2.3.3** (flora) below. With a view to these products ultimately being applied in the field, possibly by rural African communities using relatively low-tech methods, a series of experiments both in the laboratory and in the field against all mosquito life stages were carried out to examine effects on mortality and behaviour.

The three products were chosen because they are important for many reasons, many of which will be discussed in the following chapters. In addition, the simple way in which neem and fish will be tested in this thesis lends itself to field deployment, and could easily be used by communities in resource-poor rural areas. Furthermore, due to their natural characteristics, the products tested in this thesis can be used to decrease reliance on insecticides. Insecticides still have a major role to play in malaria control, but alternatives are required to reduce the selection pressure of insecticide resistance, and manage it when it arises. In addition, these tools could potentially offer an alternative to existing control methods and/or replace those when they become obsolete or ineffective.

Chapter 2 contains a review of the malaria control techniques currently available to rural African communities, places these tools in the context of an IVM strategy and discusses the role of communities in mosquito control.

In **Chapter 3,** all immature stages of *An. gambiae s.s.* were used to investigate what concentration of crude aqueous *Azadirachta indica* A. Juss (Meliaceae) (the neem tree) extract would inhibit the emergence of adult mosquitoes.

In **Chapter 4,** aqueous neem extracts were used to investigate whether the oviposition behaviour of *An. gambiae s.s.* would be significantly altered, and whether this behaviour was affected by the dose of neem used.

Chapter 5 presents the results of a fishpond census carried out in Kenya to identify whether fishponds are a major source of mosquitoes, and which characteristics were favourable to mosquito colonisation.

Chapter 6 reports on the findings from a small-scale intervention trial carried out in Kenya and run over nine months. In this trial, the edible fish *Oreochromis niloticus* L. (Perciformes: Cichlidae) was tested for its ability to control wild mosquito larvae and pupae in fishponds.

In **Chapter 7,** an application method that could be directly used in the field for the delivery of entomopathogenic fungi was tested against insecticide-resistant and insecticide-susceptible *An. gambiae s.s.* strains.

Chapter 8 reports the findings from an experimental hut trial carried out in Benin to investigate whether two species of entomopathogenic fungi could be effective against wild multi-insecticide-resistant mosquitoes.

Finally, in **Chapter 9** the results of all the preceding chapters are summarized and discussed, conclusions are drawn and recommendations made for future research.

Chapter 2

Malaria vector control options available to rural African communities in the context of an integrated vector management strategy: a review

Annabel F. V. Howard and Henk van den Berg

To be submitted in a slightly modified form

2.1 Abstract

Insecticide-treated nets have been at the centre of malaria control efforts for some time, but coverage and use in some African countries is still disappointingly low. Furthermore, insecticide resistance in malaria vectors is threatening to render such insecticide-based mosquito control tools ineffective. In response to this, the World Health Organisation promotes the use of integrated vector management (IVM) programmes for malaria control. The premise of IVM is that different control tools are used simultaneously to try and reduce disease transmission. Within these programmes there is a place for the long-established mosquito control tools that were almost completely discarded at the advent of the synthetic insecticides. The wide-scale use of environmental management, house modification and larvivorous fish has proven to be effective at reducing mosquito numbers and malaria burden, and these methods could be readily incorporated into IVM programmes. In addition, botanical larvicides, botanical repellents, and zooprophylaxis have shown some promise in small-scale trials. This review focuses on experimental findings relating to these six mosquito control tools, and details parameters that can be used to decide which tool is locally appropriate. These methods were chosen because they are readily available to rural African communities and have the capacity to be locally produced, which could lead to their sustainable use for mosquito control. The incorporation of these tools into an IVM strategy is then discussed and the need for more IVM trials is highlighted. Community involvement is important because a successful and long-lasting IVM programme will require rural African communities to monitor and evaluate mosquito breeding, and respond with locally appropriate mosquito control tools. The role of communities in IVM strategies is considered and reasons why communities do not currently employ mosquito control tactics are discussed. With adequate education and training, communities can be shown that many mosquito control tools are to be found in their own environment and these tools can be used for mosquito control relatively easily. Whilst discussing underused malaria control tools, this review is designed to

complement other IVM reviews and does not advocate relocating resources away from current malaria control methods.

2.2 Introduction

Chapter 1 introduced many of the important components of malaria including the disease dynamics and the life cycles of both the parasite and mosquito vector. In addition, the mosquito control methods currently used were highlighted, along with the problems that insecticide-based mosquito control can pose. Finally, **Chapter 1** also introduced the concept of using low-tech and natural products for mosquito control. The rest of this thesis will focus on these natural alternatives.

The reason adult mosquitoes have been targeted was explained in **section 1.6,** together with the thinking behind the growing movement towards larval control in Africa. In addition, **section 1.7.4** briefly introduced the main topic of **Chapter 2,** namely integrated vector management (IVM). IVM integrates the available resources and methods to achieve a maximum impact on vector borne disease, essentially attacking the problem from different perspectives at once (i.e. using adult and larval mosquito control and human chemotherapy). IVM was formally adopted by the World Health Organisation (WHO) in 2004 (World Health Organisation 2004b) as a strategy to improve the efficiency, effectiveness and ecological soundness of vector control. Hence IVM would decrease the reliance on chemical insecticides. Similarly, when different techniques are used in conjunction, the selection pressure of drug or insecticide resistance could be reduced. The emphasis of IVM is on examining and analyzing the local situation, utilising the appropriate mosquito control tools, and making decisions at decentralized levels; IVM cannot be implemented by the health sector alone, but requires collaboration with other public and private sectors, and the active participation of communities. Although the theory behind IVM is fairly simple, there are complex issues involved with decentralization, participation and implementation that have still not been

addressed in most African countries (van den Berg and Takken 2007, Beier et al. 2008, van den Berg and Takken 2008).

Section 1.10 argued that several natural alternatives for mosquito control exist that could be incorporated into IVM trials and programmes. Mosquitoes have been controlled for hundreds of years but many successful methods (e.g. environmental management, larval control, house modification), although discarded at the advent of synthetic insecticides in the 1940s (World Health Organisation 1982), have all proved to be effective in specific settings (Rowland et al. 2001, Awad and Shimaila 2003, Keiser et al. 2005, Ghosh and Dash 2007, Dugassa et al. 2009, Kirby et al. 2009) and could complement the use of insecticide-treated bednets (ITN) and indoor residual spraying (IRS) in IVM schemes. WHO emphasized that IVM should be evidence-based (World Health Organisation 2004b) but an inability to tease out the contributions of the "minor players" in IVM trials could lead to useful tools being overlooked or neglected and may be a reason why so few IVM trials have so far been reported. Other reasons include time and cost (Beier et al. 2008).

These simpler technologies are also cheap and can potentially be sustainable. Crucially, many of them can be produced in resource-poor rural areas of Africa where they are most needed. This is important because as discussed in **section 1.2,** when compared to urban communities, rural African communities are in general poorer (Matovu et al. 2009), less healthy (Hay et al. 2005), with less access to health facilities (Noor et al. 2003) and have more contact with malaria vectors (Kirby et al. 2008) leading to a higher malaria burden (Kelly-Hope and McKenzie 2009). Yet, rural communities would seem to have more options for vector control than their urban counterparts in terms of the use of plant and fish products, or the management of agricultural land and domestic animals. It is therefore important to investigate ways in which rural African communities can contribute to vector control using locally available materials, because involving local communities should allow for more successful and sustainable malaria control (Mukabana et al. 2006).

This chapter reviews the available evidence on malaria vector control tools that in the most part are readily available to rural African communities. The tools discussed also have the capacity to be locally produced and sustainable, and most are environmentally friendly. The methods are divided into those that affect the mosquito population size (via larval control) and those that reduce the human-vector contact. The ways these tools could be incorporated into an IVM strategy are outlined, and the problem of evidence-based decision making for vector control methods is addressed. Finally the role of communities in IVM strategies is discussed, and some of the outstanding issues that need to be addressed are highlighted.

2.3 Methods that reduce the number of mosquitoes

2.3.1 Environmental management

Two types of environmental management are environmental modification and environmental manipulation. The former refers to permanent changes that prevent mosquito breeding; in the latter the breeding sites are temporarily made unfavourable to mosquito populations (World Health Organisation 1982). Environmental management not only reduces the number of mosquitoes, but in addition the resulting extended periods needed to search for oviposition sites may affect the longevity of the adult mosquitoes by depleting their energy reserves and exposing them to more risks (Gu et al. 2006).

Managing water to control mosquitoes was first used thousands of years ago (Konradsen et al. 2004) and a systematic review of 16 trials that used environmental modification showed that the risk ratio of malaria was reduced by 88% (Keiser et al. 2005). In addition, environmental management can be long-lasting and cost-effective (World Health Organisation 1982). In the Roan Antelope copper mine in Zambia, an IVM programme with extensive environmental

modification was implemented in 1929. River banks were cleared, swamps were drained and man-made obstructions were removed in an attempt to disrupt the breeding of both *Anopheles gambiae* and *Anopheles funestus*. House screening and targeted chemoprophylaxis with quinine was also used. Not long after these measures were taken, large reductions in overall mortality rates were reported. In addition, the baseline rate of splenomegaly in children <15 years of age dropped from 36% to 6% five years after programme implementation, while splenomegaly remained at 45% in children living outside the intervention area. Furthermore, annual malaria incidence halved in the first year of the programme (Utzinger et al. 2001). Similarly, in an area of low malaria transmission in Ethiopia, environmental modification resulted in fewer mosquito-positive habitats and the numbers of anopheline larvae found were greatly reduced. The numbers of adult *Anopheles arabiensis* collected from houses was also reduced by 49% when compared to the pre-intervention data and after controlling for the reduction in the control village (Yohannes et al. 2005).

A specific example of environmental manipulation is intermittent irrigation. This is mainly carried out in rice fields and refers to temporarily draining the water and allowing the field to dry completely. Intermittent irrigation is only applicable in areas with specific soil and climate characteristics, and can reduce methane emissions from rice fields, help conserve water and significantly lower malaria vector abundance (Keiser et al. 2002). Direct effects on malaria morbidity have also been seen; in India splenomegaly (baseline 48%) and parasite (baseline 42%) rates were reduced to 4% and 0% respectively four years after intermittent irrigation was started (Knipe and Russell 1942). To test this method in Africa, Mutero et al. (2000) compared different water regimes and found that the intermittently irrigated plots of land had the lowest number of natural mosquito predators and the highest numbers of 1st instar larval *An. arabiensis*. However, when comparing the ratio of 4th instar to 1st instar larval mosquitoes, they found very low survival levels for the mosquitoes within the intermittent irrigation regime, and no significant effect on the rice yield when compared to control plots (Mutero et al. 2000).

Several parameters should be considered before the implementation of environmental management (Table 2.1). For example, knowledge about the vector's preference to breed in certain types of habitats, and information on the availability of such habitats near people's houses are crucial for planning and targeting environmental management practices. Moreover, certain wetland areas need to be preserved because they are integral to local water cycles. Clearing vegetation from streams and drains can keep the water flowing, reducing stagnant areas, and in Tanzania clearing drains was able to significantly reduce the prevalence of malaria (Castro et al. 2009). Although beneficial for mosquito control, this form of environmental manipulation can have complicated effects because clearing vegetation could change the ecological conditions of a water body and may open it up to colonisation by other types of mosquitoes that were previously unable or unwilling to breed there due to the dense covering of weeds. Similarly, drainage of swamps could lead to increased anopheline breeding because the swamps tend to house many natural mosquito predators (Carlson et al. 2004). The destruction of this ecosystem would favour the mosquitoes because they tend to colonise new or temporary water sources before their natural predators do (Service 1978).

Although environmental management can be tailored to different ecosystems and different vector breeding preferences (Keiser et al. 2005), total removal of water bodies is not desirable in many areas because people need sources of water to live and work; one Kenyan respondent even said "better the presence of mosquitoes than the absence of such water pits" (Mutuku et al. 2006a). Fifty-six percent of respondents in Kenya felt that the aquatic habitats where mosquitoes could breed were important to their lives (Imbahale et al. 2010). Nevertheless, it is possible to manage water bodies thereby minimising the risk of mosquitoes breeding. Groups of water bodies such as brick making pits are regularly found in close proximity to each other in areas such as western Kenya (Figure 2.1). All of these pits are a potential breeding habitat for local malaria vectors (Carlson et al. 2004). In such situations, practical and locally acceptable solutions of

Table 2.1. Parameters that could be used in local decision making when selecting vector control methods

Control method	Important parameters
Environmental Management	Vector breeding preference and significance of dry season refugia Habitat characteristics (size, number, stability and vector productivity) Distance to human habitations Practical considerations (local requirement for water bodies for domestic or irrigation purposes, knowledge of water management) Ecosystem and climate (e.g. soil type, likelihood of drained bodies re-flooding) Ecosystem effects (e.g. on insect mosquito predators)
Larvivorous fish	Vector breeding preference Habitat characteristics (size, number, stability and vector productivity) Distance to human habitations Lack of alternative options (e.g. environmental management) Practical considerations (availability of immature fish, market for mature fish and knowledge of fish husbandry) Suitability of water body for fish (depth, water type, stability) History of chemical larviciding Ecosystem effects (on insect mosquito predators, vegetation, indigenous fish)
Botanical larvicides	Vector breeding preference Habitat characteristics (size, number, stability and productivity) Distance to human habitations Lack of alternative options (environmental management or larvivorous fish)

	Availability of local plants with larvicidal properties Ecosystem effects (e.g. on insect mosquito predators, indigenous fish) Effects on domestic animals, human health
House Modification	Type of house construction (open eaves/door/windows) Permanence of house Proximity to vector breeding habitat Practical considerations (e.g. availability and cost of materials, knowledge of carpentry skills) Vector behaviour (endo- or exophilic biting, time of biting, anthropo- or zoophilic, resting behaviour) Human behaviour and attitudes
Botanical repellents	Type of house construction (open eaves/door/windows) Proximity to vector breeding habitat Lack of alternative options (house modification or chemical repellents) Practical considerations (availability of repellent plants, their repellent effect) Vector behaviour (endo- or exophilic biting, time of biting, anthropo- or zoophilic, resting behaviour) Human behaviour and attitudes (e.g. outside at night, compliance) Human health effects
Zooprophylaxis	Proximity to vector breeding habitat Practical considerations (availability of cattle and insecticides) Vector behaviour (endo- or exophilic biting, time of biting, anthropo- or zoophilic) Distance of cattle from human habitations; alternate hosts Prevalent zoonotic diseases

Figure 2.1. A group of brick making pits in western Kenya, each one is a potential mosquito breeding site

environmental management should be developed with active involvement of communities. For instance, in circumstances where the water is required, these pits could be merged to form one large pit which should be easier to treat. However, it is important to note that these large water bodies would need to be treated with another control tool because larger pools of water are more stable and can be more productive in terms of mosquito pupae and adults (Mutuku et al. 2006b).

A knowledge, attitudes and practices survey in western Kenya found that while 24% of respondents knew that draining stagnant water could help control mosquitoes, just 1% of them practiced this (Imbahale et al. 2010). In other areas of Kenya, communities reported using many different methods of environmental management to try and control mosquitoes (Kibe et al. 2006, Ng'ang'a et al. 2008). However, the main reasons given by those not using environmental management

were the lack of time, perceived absence of mosquitoes and a perceived lack of effectiveness (Ng'ang'a et al. 2008). This is discussed in more detail in **section 2.6**.

2.3.2 Larvivorous Fish

The deployment of larvivorous fish in appropriate water bodies has been used in mosquito control for over 100 years (Bay 1967) and can have a large impact on malaria incidence. The larvivorous fish *Gambusia affinis* (the mosquito fish) and *Poecila reticulata* (guppy) were introduced into ponds and wells in rural India and malaria declined from 73,270 cases reported in 2001 before fish introduction, to 497 cases in 2005. This reduction was attributed to the fish because considerations for the local silk worm industry meant that spraying with insecticides could not be carried out (Ghosh and Dash 2007). Despite the successes of these "mosquito fish", it is important to prioritize the use of native fish for their larvivorous properties because the introduction of non-native fish can disturb ecosystems (World Health Organisation 2002). *Oreochromis spilurus spilurus* (the Sabaki tilapia) is a native African fish that was introduced into water storage containers in Somalia over a two year trial period. At the end of the trial the number of mosquitoes caught per room had decreased from 2.3 to zero in the intervention area; in the control area the values were 3.6 and 8.9 mosquitoes per room at the beginning and end of the trial respectively (Alio et al. 1985). A similar study found that the number of mosquito larvae per water storage container was reduced by a mean of 52.8% after fish introduction (Mohamed 2003). Following this trial 83% of the community said they would accept the use of fish in their personal water storage tanks to control mosquitoes (Mohamed 2003). In Ethiopia local people had heard about the larvivorous properties of *O. spilurus spilurus* from their Somali neighbours and had taken the initiative to buy these fish to stock their water storage containers specifically to control mosquitoes (Teklehaimanot et al. 1993). The larvivorous potential of *Oreochromis niloticus* (the Nile tilapia) has been confirmed in the laboratory (Asimeng and Mutinga 1993, Kusumawathie et al.

2006) and **Chapter 6** of this thesis describes the first field deployment of this fish species specifically for malaria vector control.

Other native African fish known to be larvivorous include *Aphanius dispar* (the Arabian killifish) which is native to Ethiopia and is able to withstand polluted water (Chandra et al. 2008). As such it should be a good contender for the control of *Culex quinquefasciatus* mosquitoes, the vectors of filariasis in East Africa. It has also been used for the successful control of several *Anopheles* species; *An. gambiae* and *An. arabiensis* breeding was suppressed by 97% in a number of different breeding habitats in Djibouti (Louis and Albert 1988). Similarly, *Anopheles culicifacies adanensis* mosquitoes were successfully controlled in the port city of Assab in Ethiopia, with the fish equally effective at controlling mosquito numbers in wells, cisterns and barrels (Fletcher et al. 1992). *Nothobranchius guentheri* is native to East Africa (Chandra et al. 2008) and has eggs that are drought resistant, which would allow its use in rice fields that are intermittently irrigated or other areas where water is not constant all year round.

Louca et al. (2009) conducted a study in The Gambia that sampled native fish from the river floodplain and screened their stomach contents. Only one fish (*Ctenopoma kingsleyae*) was found to have eaten an anopheline larva. When tested in semi-field experiments, *Tilapia guineensis* (Guinean tilapia) and *Epiplatys spilargyreius* ate all late stage culicine and anopheline larvae after 24 hours. In addition, *T. guineensis* caused a 96% reduction in early stage mosquitoes after 12 days while *E. spilargyreius* caused a 69% reduction (Louca et al. 2009). While both tested fish species were clearly highly larvivorous under experimental conditions, the lack of mosquitoes in their stomachs under natural conditions caused some concern as to whether these fish could be successfully used for mosquito control in natural habitats where there would be a wide prey choice (Louca et al. 2009). This is a concern for all fish types; when farming fish they should not be overfed because this will reduce the amount of mosquitoes and natural vegetation (where larval mosquitoes hide) that they eat.

Advantages of using larvivorous fish are that they are generally self-sustaining and so ponds only have to be treated once. In rural sub-Saharan Africa where many roads are of poor quality this is a great benefit. Also, fish survival does not depend on the presence of mosquito larvae whereas other biological control agents often depend on the mosquito population not being entirely eliminated (Wright et al. 1972). Furthermore, fish can physiologically affect some *Anopheles* larvae, causing significantly prolonged developmental times and emergence as smaller adults (Bond et al. 2005). In Asia, stocking fish in rice paddies has also been shown to increase rice yield while at the same time reducing mosquito numbers and decreasing malaria transmission (Wu et al. 1991). Fish can also be more cost-effective than traditional larvicides; *P. reticulata* turned out to be more cost-effective than the organophosphate insecticide temephos when applied in the field in Sri Lanka, with the costs of temephos application 2.67 times higher than the cost of the fish (Kusumawathie et al. 2008). Of course, as with other mosquito control tools, parameters need to be considered when deciding whether or not to use larvivorous fish (Table 2.1). One disadvantage is that larvivorous fish can only be used under certain conditions conducive to their survival, and different fish species have different requirements (Trewavas 1983). They can also be difficult to transport, and the effect abandoned fish ponds have on mosquito abundance is described in **Chapter 5** of this thesis.

Effective and sustainable use of fish for vector control in Africa will require a certain element of fish husbandry. This requirement for fish husbandry should not pose a problem because small-scale fish farming has been continuously practiced in rural western Kenya for almost 50 years (Lockhart et al. 1969) and is still a favoured community activity today with people building and stocking new ponds (Figure 2.2). The 2005 Abuja Declaration on Sustainable Fisheries and Aquaculture in Africa called for the development of fish farming throughout Africa and it is conceivable that five years after this declaration many rural communities in Africa now possess the required skills to successfully rear and keep fish that can also be used for mosquito control. Unlike some other mosquito control methods, there is the added

impetus for people to keep fish because fish farming can provide a source of income and protein for people living in rural areas.

Figure 2.2. A community group in western Kenya stocking a fishpond with the edible larvivorous fish *Oreochromis niloticus*

2.3.3 Botanical larvicides

Plants naturally produce compounds to protect themselves from herbivorous insects. Due to this phenomenon plants contain compounds that can either prevent the insects feeding, slow down their growth rate, disrupt the moulting process or even cause death (Schoonhoven et al. 2005). Because of this, many plant species have been tested for their potential deleterious effects against mosquitoes; most notably for any larvicidal effects. Hundreds of plants have been screened and tested for their effects on mosquitoes; good comprehensive reviews on this subject have been written by Sukumar et al. (1991) and Shaalan et al. (2005).

Much of this research has taken place in India and Indian plants that have been shown to be mosquitocidal include *Atlantia monophylla* (Sivagnaname and Kalyanasundaram 2004), *Solanum villosum* (the hairy nightshade) (Chowdhury et al. 2008) and *Pelargonium citrosa* (Jeyabalan et al. 2003). Perhaps the best known and most thoroughly tested plant for mosquito control is *Azadirachta indica* (the neem tree) (Figure 2.3). The mosquitocidal effects of this tree have been determined both in the laboratory (Ziba 1995, Okumu et al. 2007) and field (Rao et al. 1992, Nagpal et al. 1995, Awad and Shimaila 2003) and the leaves (Siddiqui et al. 2003), fruit/seed (Batra et al. 1998, Gianotti et al. 2008) and wood (Ziba 1995) of this tree can all be used to kill mosquitoes. Field studies have also shown neem to be effective; when used as a larvicide bi-weekly in western Niger, crushed seeds caused a 49% reduction in the number of adult female *An. gambiae s.l*. (Gianotti et al. 2008). In addition, the larvicidal use of neem can reduce adult mosquito longevity (Nathan et al. 2005, Okumu et al. 2007). Neem trees already grow well in many areas of sub-Saharan Africa, they are popular shade trees (Gianotti et al. 2008), and they are also already used as a mosquito repellent in countries including Tanzania (Kweka et al. 2008), Kenya (Seyoum et al. 2002b) and Ethiopia (Karunamoorthi et al. 2009), and to treat malaria episodes in Kenya (Kibe et al. 2006). **Chapters 3 & 4** of this thesis examine the larvivorous potential of crude aqueous neem extracts, and the effects these extracts have on adult female oviposition.

Other African plants that have been shown to be larvivorous include seventeen Tanzanian plants that were screened for their larvicidal action against *An. gambiae s.s.* (Kihampa et al. 2009). In addition, three plants from Burkina Faso were able to kill field-caught *An. gambiae* and *An. arabiensis* larvae, with the eggs being more susceptible than larvae (Bassole et al. 2003). Another larvivorous native African plant is *Ricinus communis*, the castor oil plant. When collected from Sudan, aqueous extracts of this plant were able to kill the larvae of both *An. arabiensis* and *Cx. quinquefasciatus*, with *An. arabiensis* being more susceptible (Elimam et al. 2009). Unfortunately, this plant also caused an oviposition deterrence effect,

Figure 2.3. The neem tree (*Azadirachta indica*) is a popular shade tree and grows in many areas of sub-Saharan Africa; parts of this tree have been shown to have lethal effects to mosquito larvae

meaning that the adult mosquitoes selectively avoided laying their eggs in water that contained extracts of this plant (Elimam et al. 2009).

Much of the work investigating the use of plants as mosquito larvicides has used sophisticated methods to prepare extracts which, whilst producing encouraging results (Nathan et al. 2005), are impractical to produce and use in rural Africa where the lack of infrastructure requires more simple methods to be used. Furthermore, the extraction methods used can affect the bioactivity of the phytocompounds because polar solvents will extract polar molecules (Shaalan et al. 2005). Therefore, the results obtained with sophisticated extracts may not be

matched when the plants are simply placed into mosquito breeding sites. Simply placing whole plant parts into breeding sites is of added benefit because whole plants have many active and synergistic compounds meaning mosquitoes are less likely to build up a physiological resistance (Isman et al. 1996). However, as well as the inherent differences in phytochemical activity from the same plant produced in different areas (Schmutterer 1995), when these phytochemicals are placed into natural mosquito breeding sites their bioactivity may be further attenuated by the environmental characteristics of the water body and the effect of the UV light from the sun (Schmutterer 1995).

Although many plants have been shown to have larvicidal properties experimentally, African communities are not routinely using plants for larval control even though, as with the repellent plants discussed in **section 2.4.2** below, many of these plants can be grown where they are needed and could provide sustainable and relatively cheap mosquito control. Unlike botanical repellents used against adult mosquitoes, and the use of plants to kill food pests such as the cowpea beetle (Boeke et al. 2004a), there is no wide-scale use of botanical products to kill larval mosquitoes in traditional ethnobotanical practices. This may be because many people do not know where mosquito larvae can be found or what they look like (Mutuku et al. 2006a, Imbahale et al. 2010). As well as the ability to identify mosquito larvae and where they can be found, other parameters must be considered before the implementation of botanical larval control measures (Table 2.1).

2.4 Methods that reduce human-vector contact

2.4.1 House modification

In rural Africa, houses tend to be made of mud bricks with open eaves and thatch roofs, and these houses have more mosquitoes in them when compared to

concrete houses with closed eaves or metal roofs (Kirby et al. 2008). With some effort and investment these traditional African houses, that may not even have a closable door, can be modified to prevent mosquito entry. Forms of house modification include closing the eaves (Figure 2.4) and/or installing ceilings, and using curtains/screens to close off doors and windows. Closing eaves and ceilings are particularly important because trials in The Gambia and São Tomé have showed that eaves are important house entry points for *An. gambiae s.l.* but not for culicine mosquitoes (Charlwood et al. 2003, Njie et al. 2009). This is because *An. gambiae* will fly towards host odours and on contact with a vertical surface will fly upwards (Snow 1987), thus the overhanging roof directs mosquitoes towards and into the open eaves.

In Kenya, local people were encouraged to weave ceiling mats using readily available papyrus and sisal. These mats were then placed into certain houses closing off the open eaves and roof spaces. There was an 84% and 87% reduction in the odds of *An. gambiae s.l.* and *An. funestus* respectively being present in the modified houses when compared to the control, unmodified houses (Atieli et al. 2009). Similarly, an experimental hut study conducted in The Gambia compared mud-closed eaves and four different types of ceilings, to an unmodified control hut. They found that all types of ceilings significantly reduced *An. gambiae* entry with the plastic insect-screen causing an 80% reduction when compared to the control (Lindsay et al. 2003). In refugee shelters in Uganda, ceilings made of ITNs were able to significantly reduce the number of mosquitoes inside the screened shelters when compared to unscreened shelters. The authors estimated that inside a screened shelter 1 person in 1177 received a bite from an anopheline mosquito; in unscreened shelters anophelines bit 1 person in 125 (Medlock et al. 2007).

Screening windows and doorways are also important forms of house modification. Kirby et al. (2009) conducted a randomised control trial in The Gambia comparing houses where windows and doors were screened with netting and the eaves had been filled in (full screening), with houses where netting ceilings had been installed

(screened ceilings). They found that either screening method reduced *An. gambiae s.s.* house entry by about a half when compared to unscreened control houses. The estimated entomological inoculation rates (EIR) showed that the screened ceilings had half the EIR compared to the control houses, whereas the EIR in the fully screened houses was a third of the controls. Also, children living in screened houses had significantly higher haemoglobin levels when compared to children from the control houses, and significantly fewer cases of anaemia (Kirby et al. 2009). When offered, the vast majority of people chose the more complete house modification because it was more able to reduce the entry of other pests, improved the attractiveness of their house and improved their privacy (Kirby et al. 2009).

House modification is becoming more utilised although this varies from area to area. While only 3% of people in a survey in rural Kenya had screened doors/windows (Ng'ang'a et al. 2008), 80% of monitored households in Dar es

Figure 2.4. Closing off the eaves of a traditional rural house can prevent mosquito entry

Salaam had installed window screening and almost 80% had either a ceiling or closed eaves or both (Ogoma et al. 2009). It appears that houses become progressively more mosquito-proof with increasing income. One of the challenges is to break this association through mosquito-proofing rural houses. Where house modification has not been taken up, the reasons given by various African communities for not already closing the eaves of their houses included the high cost of procuring the materials (Ogoma et al. 2009), the extra effort required (Lindsay et al. 2003) and the need for air circulation to combat the high daytime temperatures (Atieli et al. 2009). A study in Tanzania asked people who had not modified their houses how much they expected to pay, in general people tended to overestimate the cost of house modifications (Ogoma et al. 2009).

In The Gambia, full house screening with locally available netting was estimated to cost US$11.11 per person treated (Kirby et al. 2009), while ceilings had an estimated cost of £0.36-0.59/person/year (Lindsay et al. 2003). Papyrus mats were made in Kenya from locally available materials and sold for US$1 each for use as ceilings, with several mats being needed for each ceiling depending on house size (Atieli et al. 2009). This relatively high initial cost is a disadvantage of house modification, as is the reduced air flow, but on the plus side maintenance should be cheap and simple and the effects are long-lasting. House modification could be especially worthwhile and economically viable if there are a large number of people sleeping within one house where each of those people cannot afford ITNs; money spent on window screening in Tanzania was almost the same per person as that spent on ITNs (Ogoma et al. 2009). House modification can also directly impact malaria incidence. A review of eight studies showed that house modification reduced the risk of malaria by 79.5% (Keiser et al. 2005). Another benefit of house modification is that it can protect the whole household. This is especially important in refugee situations where it is not possible for everyone to have an individual ITN (Medlock et al. 2007).

2.4.2 Botanical repellents

Various plants can be used to repel mosquitoes from entering houses. Although normally the most commonly deployed method (Seyoum et al. 2002b), it is not just burning plants that can cause repellency. In Kenya some communities place whole plants or tree branches in their houses (Seyoum et al. 2002b), and a field study found that live potted plants could significantly repel *An. gambiae s.l.* but not *An. funestus* (Seyoum et al. 2003). In Eritrea hanging the fresh leaves of *Ocimum forskolei* (sweet basil) around the bed led to a 53% reduction in the numbers of *An. arabiensis* found in the house (Waka et al. 2004). A questionnaire in Ethiopia identified several different ways people used the repellent plants, such as spraying aqueous solutions or laying the leaves on the floor (Karunamoorthi et al. 2009) and it has been reported from Nigeria that farmers place leaves on their roof to repel mosquitoes (Oladepo et al. 2010).

In Kenya and Tanzania a range of plants are traditionally used to repel mosquitoes from houses including *Ocimum* spp. (wild basils), *Eucalyptus globules* (the blue gum tree), *Tagetes minuta* (the Mexican marigold) and *Lantana camara* (wild sage) (Figure 2.5) (Seyoum et al. 2002b, Kweka et al. 2008). *Lantana camara* is a shrub that grows in many places in Africa. Live *L. camara* plants have been shown to repel *An. gambiae s.l.* mosquitoes from house entry (Seyoum et al. 2003). Similarly, 42% and 29% repellency of *An. gambiae s.s.* was seen when the leaves or seeds respectively were thermally expelled (heated on thin metal plates over charcoal); in contrast, when the leaves and seeds were directly burned no significant repellent effects were seen (Seyoum et al. 2002b). Laboratory evaluations were undertaken to test whether certain Ethiopian plants were really effective at repelling mosquitoes. Of those tested, burning the roots of *Silene macroserene* was found to be the most repellent, driving 94% of *An. arabiensis* mosquitoes away. The least repellent plant tested, *Olea europaea* (the olive tree), was still able to drive away 80% of the mosquitoes (Karunamoorthi et al. 2008). In the laboratory, *Ocimum suave* caused 81% of *An. gambiae s.s.* and 89% of *An.*

arabiensis to be repelled from seeking a blood meal in a tunnel test setup (Kweka et al. 2008). *Ocimum kilimandscharicum* (camphor scented basil) produced similar levels of repellence and both plant species were also effective at repelling the nuisance mosquito *Cx. quinquefasciatus* (Kweka et al. 2008). Semi-field studies using *Ocimum* spp. found that when burned alone, both *Oc. suave* and *Oc. kilimandscharicum* caused 26-28% and significant repellency of *An. gambiae s.s.*. When they were thermally expelled this repellency was increased to around 50% for both plants. However, when the two plants were thermally expelled together or in combination with *L. camara,* the repellency was just 15% and 4.6% respectively, neither result being significantly different from the control (Seyoum et al. 2002b). Plants have also been shown to effectively repel wild mosquitoes in field trials. A field study evaluating four plants in Eritrea compared two methods; directly burning the plants and thermal expulsion (Dugassa et al. 2009). They found that different

Figure 2.5. *Lantana camara* is a ubiquitous shrub commonly used as a mosquito repellent in Africa countries

plants were more effective at repelling *An. arabiensis* than *Anopheles pharoensis* and vice versa. For all plant types and both application methods there was significant mosquito repellency when compared to just burning charcoal. When the plants were directly burned there was 65-73% repellency of *An. arabiensis* and 66-73% repellency of *An. pharoensis*. Thermal expulsion repelled 72-79% of both types of mosquito but this was not significantly different from the directly burning results (Dugassa et al. 2009). In Kenya, *Corymbia citriodora* (lemon scented gum tree), *Oc. suave* and *Oc. kilimandscharicum* were thermally expelled in mud-walled, grass-thatched houses. All three plants significantly repelled *An. gambiae s.l.*, and even showed a degree of residual activity; the level of *An. gambiae s.l.* repellency found was similar to that caused by a commercially available slow-burning mosquito coil (Seyoum et al. 2003). Despite these encouraging results with *An. gambiae s.l.*, only *Oc. kilimandscharicum* was able to significantly repel *An. funestus* (Seyoum et al. 2003).

Repellent plants are regularly used by African communities. In Burkina Faso plants are burnt in both the bedroom and living areas to repel mosquitoes (Yamamoto et al. 2009). In The Gambia, burning churai (perfumed woods mainly from the tree *Daniellia oliveri* (the African copaiba balsam tree)) can significantly reduce the presence of mosquitoes within a house, with a 44% decrease in the odds ratio compared to houses that did not burn churai (Kirby et al. 2008). However, the burning of churai had no impact of the incidence of malaria in children (Snow et al. 1987). A knowledge and usage questionnaire in northern Ethiopia identified fifteen local plants regularly used to protect against insects/mosquitoes (Karunamoorthi et al. 2009). Even though 44% of the respondents were illiterate, 97% of the people questioned were able to identify the plants considered most useful for repelling mosquitoes (Karunamoorthi et al. 2009), indicating that ethnobotanical knowledge is not limited by a lack of formal education. People came from varying socio-economic backgrounds but those earning the least money per month were significantly more likely to use plants as repellents when compared to those on higher incomes (Karunamoorthi et al. 2009).

Indigenous repellent plants can be found for free in many areas of Africa. They can be grown relatively easily allowing their sustainable use for mosquito control. In addition, they could be cultivated and sold, bringing in a source of income to some industrious people. The use of repellent plants can protect all inhabitants of the house, and can even be used to protect people outdoors. As with the other tools reviewed in this paper, there are certain parameters that should dictate whether or not to use botanical repellents (Table 2.1). Disadvantages include the need for frequent applications and almost no residual effect. Also, wind and rain could decrease the effectiveness, and the repellent properties of the plants may differ from plant to plant as with the larvicidal properties (see **section 2.3.3** above). Finally, the use of repellents possibly reduces the perceived urgency of other, perhaps more effective, measures of self protection such as house screening or the use of ITNs.

2.4.3 Zooprophylaxis

Zooprophylaxis is defined as “the use of wild or domestic animals, which are not the reservoir hosts of a given disease, to divert the blood-seeking mosquito vectors from the human hosts of that disease” (World Health Organisation 1982). Zooprophylaxis can be passive or active. In passive zooprophylaxis the effect comes from animals that are already kept by a household (Figure 2.6); active zooprophylaxis refers to the active deployment of animals or a change in the way in which they are kept specifically for a zooprophylactic effect. The success of zooprophylaxis in an area depends on the selectivity of the local mosquito species and the level of zoophily (preference for biting animals). For example *An. arabiensis* is an important malaria vector in many African countries and is strongly zoophilic, and more specifically, more attracted to cattle than other domestic animals (Mahande et al. 2007a). Whereas, *An. gambiae s.s.* is widely regarded as the most efficient malaria vector in sub-Saharan Africa because it is highly anthropophilic (preference for biting humans) (Costantini et al. 1999).

Figure 2.6. Cattle are regularly kept close to homesteads and under some circumstances can be used to protect humans from mosquito bites

Studies in Tanzania (Mahande et al. 2007a) and Kenya (Kaburi et al. 2009) have shown protective effects of using cattle for zooprophylaxis. In Tanzania, human blood indices (HBI) of *An. arabiensis* were lower when collected from houses with cattle than from houses without cattle (Mahande et al. 2007a). In Kenya, households with less than five cattle and that owned a bednet had increased protection from mosquito bites, although they found that the level of protection decreased as the number of cattle increased (Kaburi et al. 2009). Zooprophylactic successes have also been reported when the cows had been treated with insecticide. Pyrethroid-treatment of cattle is already widely used in Africa and other areas of the world for the control of vector-borne diseases. If an insecticide-treated cow is used for zooprophylaxis then humans can be protected because the

mosquitoes that come into contact with the cow will not be diverted onto humans (Hewitt and Rowland 1999, Habtewold et al. 2004) but will die of the insecticide (Rowland et al. 2001). In Tanzania, cattle treated with deltamethrin were able to increase the mortality of *An. arabiensis* (Mahande et al. 2007b), and in a trial in Asia, the zoophilic vectors *Anopheles stephensi* and *An. culicifacies* were more than 46% less abundant in villages where cattle had been treated with deltamethrin (Rowland et al. 2001). In addition, this community-randomized trial showed that treating cattle with deltamethrin substantially decreased the incidence of malaria caused by *Plasmodium falciparum* and/or *Plasmodium vivax* (Rowland et al. 2001).

Unfortunately, failure of zooprophylaxis has been reported in areas where the main malaria vectors are anthropophilic (Bøgh et al. 2002, Kirby et al. 2008) and zoophilic (Seyoum et al. 2002a, Habtewold et al. 2004). In addition to these recorded failures, other disadvantages of zooprophylaxis are that for mosquito populations with a high searching-related vector mortality rate, the expected beneficial effect of zooprophylaxis could be reversed because the number of mosquitoes in an area could actually be increased by the cattle providing more blood meals. Also, more adult mosquitoes could survive the duration of the parasite extrinsic incubation period (EIP) and therefore become infectious (Saul 2003). Finally, when the mosquito breeding sites are situated far from human and animal habitations it is thought that the animals may actually attract the mosquitoes to the humans (Saul 2003).

Successes and failures of zooprophylaxis have been reported and it appears that it is most effective when the cattle are treated with insecticide. One major drawback for the use of zooprophylaxis in Africa is the dependence on the biting preference of the local vector species. The highly anthropophilic nature of *An. gambiae s.s.* rules out effective zooprophylaxis in many areas of sub-Saharan Africa. Evidence on key parameters (Table 2.1) and a strong linkage between research and extension will be critical for future implementation of zooprophylaxis

2.5 How to combine methods in the context of IVM?

So far, six types of methods that could be adopted by rural communities to supplement mainstream malaria control interventions have been outlined. Mathematical models have predicted that combinations of some of these methods would be much more effective than the individual effects (Saul 2003, Yakob and Yan 2009). For instance, zooprophylaxis is expected to be more effective when the number of mosquito breeding sites is reduced through environmental management (Saul 2003). As well as the hypothesised effects, these methods have already proved effective in field trials in specific settings. When fish were used with IRS, early detection and prompt treatment in India, malaria was successfully controlled (Singh et al. 2006). Furthermore, when fish were combined with a microbial larvicide the fast but not prolonged effect of the microbial larvicide at reducing mosquito numbers was complemented by the slow but sustained effect of the fish (Hurst et al. 2006). Environmental management has been used successfully in IVM programmes that combine mosquito control methods in Zambia (Utzinger et al. 2001, Chanda et al. 2008), Kenya and Tanzania (Mukabana et al. 2006) and India (Rajagopalan and Panicker 1985). In addition, zooprophylaxis appears to have an additive effect when used with long lasting ITNs (Kaburi et al. 2009). The World Health Assembly resolution WHA 50.13 calls on Member States to “support the development and adoption of viable alternative methods of controlling vector-borne diseases and thereby reduce reliance on insecticides” (World Health Organisation 2004b). Despite this, some of the methods discussed above have not been the subject of recent IVM trials even though they all have benefits that could be exploited for mosquito control.

IVM trials should look to examine three categories of evidence; evidence about the effects of an IVM strategy, evidence about the effectiveness of individual methods, and evidence about the parameters that determine the effectiveness of each method. The first two categories demand large scale trials, which have been scarce. There have been various studies reporting the effects of combinations of

methods, but in most cases the study design did not allow for teasing out the effect of each method to determine whether there has been an additive or synergistic effect. Moreover, the measured effectiveness could be location-specific due to the influence of local parameters. IVM trials using a range of techniques need to be carried out so that the relative importance of various mosquito control tools and the incremental effect of methods when used in combination can be verified. In addition, they need to look beyond the entomological outcomes and report on the impact on malaria transmission, morbidity and mortality. When deciding which control methods to use locally or to test in IVM trials, evidence on the parameters that determine the effectiveness of each tool (Table 2.1) should be considered with reference to the local situation (World Health Organisation 2004b). For example, local knowledge about preferred breeding habitats, population dynamics and behaviour of the vector provides evidence to aid decision making on vector control, even though the effect on disease may never be established locally. In addition, the methods discussed above may suit certain African communities more than others. For instance nomadic tribes tend to have many cattle but are less likely to invest in permanent house modification since they move and rebuild their houses relatively frequently. In the same vein, lake-side communities tend to be fishermen and may prefer to use larvivorous fish. In addition to this, communities judge mosquito control tools on how effective they can be in the short term (Ng'ang'a et al. 2008). Therefore, methods like environmental management that have more of a long term effect should be combined with methods that reduce mosquito biting in the short term.

All of the above methods can be combined with most other mosquito control techniques (like ITNs, IRS etc), however, there are some methods that are incompatible. For instance larvivorous fish can be adversely affected by some chemical larvicides. *Gambusia affinis* is highly susceptible to a range of chemical insecticides and herbicides (Walton 2007). In Sri Lanka the insecticide fenthion is regularly sprayed for mosquito control. Even after the first application of fenthion the condition of *O. niloticus* fish had decreased by around 20% when compared to

pre-treatment levels; subsequent insecticide exposure further reduced the condition of the fish (Jayasundara and Pathiratne 2008). Similarly, the swimming speed of the rainbowfish *Melanotaenia duboulayi* was significantly reduced after exposure to the insecticides temephos and pirimiphos-methyl, but not after exposure to *Bacillus thuringiensis* var. *israelensis* (Bti) (Hurst et al. 2007). Fish are also not always compatible with natural predator conservation because they are likely to eat the aquatic invertebrates (el Safi et al. 1985, Louca et al. 2009). Natural predator conservation may also not be very compatible with environmental management because if the number of water bodies is decreased then necessarily this will impact the aquatic predator populations, especially if older and more stable habitats like swamps are targeted (Carlson et al. 2004). Neem has been shown to adversely affect many species of fish including *G. affinis* (El-Shazly and El-Sharnoubi 2000, Awad 2003). Other plant-derived larvicides could also be incompatible with fish, chemical and microbial larviciding and/or natural predator conservation, however, more research needs to be carried out to examine this. Despite these restrictions, the tools discussed in this review have many benefits that are discussed individually in the sections above. An additional benefit is that most of the methods do not require insecticides and as such they could be of particular use in areas where insecticide resistance is rendering tools such as ITNs ineffective (N'Guessan et al. 2007). In addition, these methods could play an important role in areas where there are low levels of malaria transmission and where elimination is a distinct possibility (Beier et al. 2008).

2.6 The role of communities in IVM

For mosquito control to be sustainable it must be economically and ecologically feasible and interventions should be culturally acceptable (Ng'ang'a et al. 2008). Successful IVM programmes will require involvement from several sectors ranging from the ministerial level to the community level (van den Berg and Takken 2007,

Beier et al. 2008, Chanda et al. 2008) because the principle of IVM is that effective control will cease to be the preserve of the health sector and community participation will be required. Most malaria vectors are closely associated with human habitation and, as a consequence, many of the risk factors of malaria disease are located within people's own sphere of influence. The health sector or programme implementers lack ready access to this peridomestic environment and this is why communities have an important role to play in the development of IVM strategies; they can assume responsibility and take control of their environment in order to reduce malaria risk factors. In addition, it is thought that community participation will allow more accurate and timely targeting of control interventions (van den Berg et al. 2007). Community participation is also important because their actions can lead to an increased likelihood of disease transmission (van den Berg and Takken 2007, Mutuku et al. 2009). Nevertheless, it is not always easy to motivate people into action for a number of perfectly valid reasons.

In general, communities do not employ mosquito control methods for three reasons. Firstly, they don't have the time or money available and need to be doing things that will give greater (usually economic) returns (Kibe et al. 2006). In other words, the people who are most at risk of malaria have competing interests for their time and money, and cannot always prioritise mosquito control (Rajagopalan and Panicker 1985, Ng'ang'a et al. 2008). A survey in Kenya found that 77% of people said they lacked the time to regularly drain mosquito breeding sites and 68% said they were unable to afford mosquito control tools (Ng'ang'a et al. 2008). In addition, people with little money generally only have access to the most basic malaria control tools; a survey in Ethiopia found that people with less money were significantly more likely to use plants as repellents (Karunamoorthi et al. 2009). Mosquito control is more likely to be sustainable if it is linked to socioeconomic growth (Rajagopalan and Panicker 1985, Kibe et al. 2006) and many of the methods discussed in this review have the potential to be farmed where appropriate and could become economically viable.

The second reason is a lack of adequate knowledge about what to do and how to do it (Kibe et al. 2006, Mukabana et al. 2006), although this varies from area to area. In some areas of Kenya and Tanzania, communities were trying to combat malaria themselves but they did not have access to the correct information (Mukabana et al. 2006). However, in another area of Kenya just 6.5% of people said they lacked the know-how to implement mosquito control tools (Ng'ang'a et al. 2008). Many communities in Africa know that malaria is transmitted through mosquito bites (Kibe et al. 2006, Ng'ang'a et al. 2008, Imbahale et al. 2010, Oladepo et al. 2010), and 83% of respondents in a cross sectional survey were able to identify mosquito breeding habitats (Ng'ang'a et al. 2008). People also know that mosquitoes breed in man-made habitats, and they were aware of their own role in habitat creation (Mutuku et al. 2006a, Oladepo et al. 2010). However, whilst knowing where mosquito larvae breed, many people do not know what they look like (Mutuku et al. 2006a, Imbahale et al. 2010). Where community members have been taught the specifics of *Anopheles* mosquito ecology and biology, such as where to find them and what they look like, they have been very interested (Mukabana et al. 2006). Nevertheless, a recent survey in Tanzania carried out to assess the ability of trained community representatives to correctly identify mosquito breeding habitats found that they located just 97 of the 230 anopheline-positive habitats (the rest were found by the assessor). Furthermore, the community representative correctly identified just 29 of these 97 habitats as being anopheline-positive (Chaki et al. 2009). Any successful IVM strategy will require community-targeted education if communities are to be directly involved in the evaluation and monitoring of the mosquito situation and in control tool deployment. Even basic training would allow identification of the more prolific mosquito breeding habitats and would in turn allow targeted mosquito control activities. Nevertheless, results from Tanzania indicate that even after training, surveys should be conducted to verify how efficiently community members can correctly identify mosquito habitats (Chaki et al. 2009).

The third reason communities do not use mosquito control tools is a general lack of

incentive because communities feel that they need outside assistance (Mutuku et al. 2006a). In an area of Kenya, public health officers and the local administration had previously enforced measures of environmental management that the communities then carried out for fear of legal reprisal. It appears that in this area, the community is unwilling to participate in environmental management without legal enforcement (Ng'ang'a et al. 2008). Similarly, in another area of Kenya, communities felt that assistance from government or donors was a prerequisite for them to participate in environmental management (Mutuku et al. 2006a). Another study found that younger respondents were more likely to believe it is up to the community to do mosquito control, with older respondents feeling it was the government's job (Imbahale et al. 2010). This study also compared rural and urban attitudes and found that those in the urban area were significantly more likely to feel that the government should take the lead in mosquito control (Imbahale et al. 2010). In Malindi, a town in Kenya, community representatives feel that the Municipal Council is mandated to conduct vector control because they collect taxes (Kibe et al. 2006). In Nigeria, farmers felt that the government should enforce policies and deploy people to promote environmental sanitation (Oladepo et al. 2010) and in Tanzania, community members wanted financial incentives before participating in environmental management (Castro et al. 2009). Incentives are not always the answer, as will be seen in **Chapter 5**. This lack of motivation and ownership is a problem when decentralising decision making for mosquito control because communities are used to being told what to do by authoritative figures and are used to control activities happening without their direct involvement.

Despite these reasons, communities can see the benefit of these mosquito control tools. In Tanzania, communities wanted environmental management to be added to the national priority interventions as a component of IVM (Mukabana et al. 2006). A Kenyan community widely advocated larviciding, although they also recognised the financial constraints involved (Mutuku et al. 2006a), and members of another Kenyan community regularly carry out vector control activities, although this contribution is decreasing since funding was withdrawn (Kibe et al. 2006).

Furthermore, 93% of community members were willing to participate in mosquito control in western Kenya (Imbahale et al. 2010). In addition, community-based mosquito control programmes have been successfully implemented in many African countries. In Dar es Salaam, Tanzania, a community-based larval control programme has been operating for a number of years (Fillinger et al. 2008, Chaki et al. 2009). In Cameroon, an IVM trial found that "the implementation, acceptability and ownership of the programme was undoubtedly enhanced by using community-based vector team members rather than house-holders having to allow strangers to enter their houses" (Matthews et al. 2009). Finally, Zambia has successfully implemented a country-wide IVM programme that trained community members in specific mosquito control techniques. Community awareness was stimulated by enhanced advocacy, social mobilization and the availability of legislation and the programme has resulted in lower malaria incidences in all districts (Chanda et al. 2008).

Effective communication, education and training strategies are required to provide communities with the correct information to be able to accurately assess and act upon the threat of mosquito breeding in their peridomestic environment. Several strategies have been developed and used to increase people's knowledge and motivation with the aim of changing their behaviour. Mass media reach a wide audience but rely on simple messages that do not easily change people's behaviour. The strategy of Information, Education and Communication (IEC) uses a combination of informational, educational and motivational interventions to change people's attitudes and behaviours but, despite having demonstrated an effect on knowledge and attitudes, the impact on behaviour has been less clear. Communication for Behavioural Impact (COMBI) is a campaign-like strategy that has demonstrated a more clear impact on human behaviours in relation to vector-borne disease, including malaria (World Health Organisation 2004a). This strategy aims to achieve some specific behavioural changes using public relations, advertising, and interpersonal communication.

A step further from changing people's behaviour is the strategy to empower people to take more control over their lives to contribute to improving their situation. It has been suggested that empowerment can only take place when two basic conditions are met (Bartlett 2008); the necessary means, or enabling factors, should be in place, and a process of analysis and decision making for subsequent action should be adopted. Hence, empowered people are able to purposely adapt their behaviour to prevailing circumstances. An example of a strategy of empowerment is the Farmer Field School. In many areas of the world, farmers attend these schools that teach them in an informal setting all about integrated pest management (IPM) and agricultural practices (van den Berg and Knols 2006). In Sri Lanka, IVM was integrated into the Farmer Field School curriculum which led to farmers making locally appropriate vector control decisions involving many different control methods (van den Berg et al. 2007). Farmers have a vested interest in mosquito control because not only do they create more mosquito breeding grounds by their activities (Chaki et al. 2009, Mutuku et al. 2009), but mosquito numbers and malaria incidence can peak at agriculturally important times which can reduce the available work force. Furthermore, in the rural areas of sub-Saharan Africa where the climate and geography are favourable for malaria transmission, most families are farmers (Ng'ang'a et al. 2008) and they have skills that lend themselves to use for mosquito control (such as water management) and some of the control techniques discussed above (fish and plants) could be cultivated on a small-scale in the areas required.

The methods discussed in this review further lend themselves to community deployment because of their relative ease of access. For instance, the methods with a biological component (zooprophylaxis, fish, and plants) are already in situ in many areas. Cattle are kept by many rural African communities as forms of transport, providers of milk and as a measure of their wealth. Fish farming is widespread (FAO Inland Water Resources and Aquaculture Service 2003, WorldFish Center 2005) and many of the plants identified as larvicides or repellents can easily be found. The selection of these methods and the way they are used

needs to be adapted by people to their local situation according to certain parameters (Table 2.1) as opposed to ITNs which are applicable (almost) everywhere. Communities need to be educated, empowered and trained so that they realise that many mosquito control tools can be found in their own environment, and can be used for mosquito control relatively easily.

2.7 Conclusions

This review highlights often overlooked methods that could be used in an IVM setting and/or by communities. IVM programmes need to be sustainable, economically viable and community focussed and the control methods outlined in this review could help IVM programmes fulfil these requirements. Due to their relative ease of use and, in some parts, biological nature, the above mentioned techniques could be implemented directly by communities to help themselves to control mosquitoes and reduce disease transmission. To our mind, the best scenario is one in which these mosquito control tools can be produced by local communities in the setting where they are needed, negating the burden of poor infrastructure. This would mean that not only will there be sustainable vector control, but also a source of income to those people in the resource-poor areas. Hopefully, this will in turn allow them to afford other control tools such as ITNs and anti-malarial drugs.

Lots of research funding is addressing the problem of malaria with high-tech solutions. However, solutions such as vaccines and genetically modified/sterile mosquitoes are unlikely to become operational within the next few years (Ballou 2009, Catteruccia et al. 2009, Greenwood and Targett 2009, Townson 2009). Whilst these high-tech solutions are badly needed, methods such as those discussed above are already available. These methods have proven to be effective in specific settings, and we have specified parameters to assist in decision making as to whether a method is likely to be effective in a particular setting. For all of the

methods discussed here, more research is urgently needed on the parameters that determine the effectiveness in the field. In the spirit of IVM and utilising all available mosquito control tools that are deemed locally appropriate, many of the above methods could be incorporated into IVM programmes. While these methods may not be as effective as the current insecticide-based tools in use (ITNs, IRS), and should not compete with ITNs and IRS for funding, with adequate education and awareness creation stakeholders in malaria control may decide to complement the more effective mosquito control tools with the methods discussed in this review.

2.8 Acknowledgements

We would like to thank Matt Kirby for letting us use the house modification photo (Figure 2.4). We would also like to thank Willem Takken, Marcel Dicke, Jo Ross and Vicky Howard for comments on a previous version of this review.

Part I

FLORA

EXAMINING THE EFFECT OF *AZADIRACHTA INDICA* (THE NEEM TREE) ON A MAJOR MALARIA VECTOR

Chapter 3

Laboratory evaluation of the aqueous extract of *Azadirachta indica* (the neem tree) wood chippings on *Anopheles gambiae s.s.* mosquitoes

Annabel F. V. Howard, Elizabeth Adongo,

Ahmed Hassanali, Francois Omlin, Anthony

Wanjoya, Guofa Zhou, John Vulule

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3.1 Abstract

Azadirachta indica A. Juss (the neem tree) is a source of limonoid insect growth regulators and grows well in many places in sub-Saharan Africa. We explored the potential of using neem wood and bark chippings in malaria vector control by evaluating their aqueous extracts as a larvicide and growth disruptor of *Anopheles gambiae s.s.* (Diptera: Culicidae) under laboratory conditions. Immature stages of the mosquito were tested using WHO guidelines. Fifty percent inhibition of adult emergence (IE_{50}) of all larval instars was obtained with less than 0.4 g of neem chippings in one litre of distilled water. For pupae, significant mortality occurred at 5 g/l. Inhibition of pupation was seen with some larvae staying as LIVs for nine days before dying. In addition to growth retardation, reduced reaction by larvae to visual and mechanical stimuli observed at higher neem concentrations may make them more susceptible to natural predators. There were no significant differences in the sex ratio of emerged adults or wing length of females when compared to the controls. High Performance Liquid Chromatography of aqueous extracts revealed a series of constituents of varying polarity, including the limonoids nimbin and salannin which were quantified. Azadirachtin was not detected and the observed activities are attributed to other constituents of the chippings. Such larvicides can be particularly effective where larval habitats are relatively large and readily identifiable. Aqueous extracts of neem wood chippings can be produced locally and their use has the potential to be a low-tech component of integrated malaria vector control schemes in sub-Saharan Africa.

3.2 Introduction

Due, in part, to rising drug resistance of the parasite, vector control is considered the most feasible way of controlling malaria in Africa today (Trape et al. 2002). Current vector control methods target the adult mosquitoes aimed at reducing the

vectorial capacity in an area (MacDonald 1957, Garrett-Jones 1964). However, the effectiveness of these control methods can be reduced by behavioural changes of the adult mosquitoes (Pates and Curtis 2005). Larval control is an often overlooked control method that can be extremely useful either by itself or in an integrated vector management (IVM) programme (World Health Organisation 2004b) (**Chapter 2**).

Killeen et al. (2002) suggest that the limitations of larval control in sub-Saharan Africa are 'practical rather than functional', and that due to the limited mobility of immature mosquito stages they can be effectively controlled. Several studies have shown that relatively large water bodies harbour mosquito larvae in western Kenya (Lockhart et al. 1969, Fillinger et al. 2004, Mutuku et al. 2006b, Howard et al. 2007, Howard and Omlin 2008) (**Chapters 5 & 6**) and, presumably, also elsewhere in Africa where rural populations are increasingly putting more pressure on the land. Although these mostly man-made habitats have been found to be more productive in terms of pupal production than the "traditional" *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae) habitats (such as hoof prints and tyre ruts) (Mutuku et al. 2006b), larval control is widely considered to be too labour intensive in sub-Saharan Africa. However, new tools exist to easily identify such habitats (Mushinzimana et al. 2006) that can facilitate targeted larval control.

Concerns about chemical insecticides and their persistence in the environment, as well as development of physiological resistance in the insects, have stimulated the search for eco-friendly larvicides. This is in line with section 2.4 of the 1997 World Health Assembly resolution 50.13. *Azadirachta indica* A. Juss (the neem tree) has well known insecticidal (Raghunatha Rao et al. 1988, Wandscheer et al. 2004) and insect growth regulatory (IGR) constituents (Sukumar et al. 1991, Batra et al. 1998, Copping and Menn 2000) and has been used for centuries in India (Schmutterer 1995).

Despite the many investigations into the mosquitocidal properties of neem leaves and fruit (Rao et al. 1992, Nagpal et al. 1995, Batra et al. 1998, Mulla and Su 1999,

Awad and Shimaila 2003, Siddiqui et al. 2003, Wandscheer et al. 2004), little work has been done using the wood/bark, with only one brief report on its effect on *An. gambiae* larvae (Ziba 1995). Neem grows well in arid tropical and subtropical areas but does not do well at altitudes >1,000 m (Schmutterer 1995).

We are searching for an effective, long lasting and natural insecticide that can be cheaply implemented in rural African settings. This paper reports the results of a crude aqueous extract of *A. indica* wood and bark chippings as a larvicide and growth regulator of *An. gambiae s.s.* Kisumu strain mosquitoes under a controlled laboratory setting.

3.3 Materials and Methods

3.3.1 Mosquitoes

The Kisumu strain of *An. gambiae s.s.* was used. This strain has been maintained as a colony at the Kenya Medical Research Institute (KEMRI), Kisumu for 14 years. All four larval instars (hereafter called LI, LII, LIII, and LIV as appropriate) plus pupae were used in the experiments because phytochemicals can affect different life stages to varying degrees (Sukumar et al. 1991). For the LIV used, specifically early stage LIV larvae were used and the pupae were less than 24 hours old when tested.

3.3.2 Preparation of aqueous insecticidal extracts

Wood and bark from neem trees collected from Mbita Point in western Kenya were fed into a basic wood chipping machine to produce wood chippings (roughly 1 x 3 x 0.2 cm; see photo on page 87 (not to scale)) which were left to dry in the shade. These chippings were soaked in distilled water for five days and then removed by filtration, leaving the aqueous extract into which the neem phytochemicals had

leached. The different concentrations were made by serial dilution using the filtrate. New neem extracts were made for each replicate. Distilled water was used for the controls. *Bacillus thuringiensis* var. *israelensis* (Bti) from VectoBac® DT tablets (Valent, Libertyville, IL) at a concentration of 520 International Toxic Units (ITU) per 500 ml distilled water was used for comparison in the first set of experiments.

3.3.3 Experiment one

Five hundred ml of the treated water was placed in 1 litre white plastic bowls (water depth of 2.5 cm). Twenty-five mosquitoes of the same instar for the five immature stages (LI-LIV plus pupae) were exposed to each of the water types (control, different neem concentrations and Bti) in six replicates. The neem concentrations used corresponded to 100, 10, 1, and 0.1 grams neem wood per litre water. All bowls were covered with netting. The larvae were fed on 'AniCare' fish food every 24 hours. The mosquitoes were checked at 6, 12, 24, 48 hours, and thereafter every 24 hours until all the mosquitoes had either died or emerged. Emerged adults were sexed and removed. The larvae and pupae were checked by disturbing the surface of the water, any not returning to the surface were considered dead and removed (World Health Organisation 1981). The dead mosquitoes exposed to neem were then examined under a dissecting microscope to identify any morphological abnormalities.

Probit analysis of the experiment one data was used to work out neem concentration ranges for each larval instar that would allow accurate calculation of concentrations that gave 50% and 90% inhibition of adult emergence (IE_{50} and IE_{90} respectively) (World Health Organisation 2005). These neem concentrations were used in experiment two (described in **section 3.3.4** below). The concentrations ranged from 0.0125 g/l to 0.8 g/l for LI, LII and LIII. For LIV, the 0.2 and 0.8 g/l concentrations were substituted by 0.4 g/l and 1.6 g/l respectively. For pupae, the range was 5 to 180 g/l.

3.3.4 Experiment two

WHO guidelines for testing insect growth regulators were followed with slight modifications (World Health Organisation 2005). Specifically, this entailed exposing immature mosquitoes to a range of concentrations of aqueous neem extracts and control water in cups. The modifications included checking the cups every 24 hours and 500 ml cups (water depth 5.5 cm) were used to allow sufficient room for any emerging adults. Also, more food was given to prevent cannibalism (Koenraadt and Takken 2003). As in experiment one, 25 larvae of each immature stage were exposed to each water type and the cups were covered with netting. Each treatment was replicated five times. We found very high control mortality when pupae were exposed to distilled water. We therefore ran five replicates with the purified water 'Dasani ^{TM}'. We used the purified water for both the control water and we soaked neem in it, so that neem remained the only variable in the experiment.

During all experiments the mosquitoes were kept under a natural 12L: 12D light-dark cycle and the mean (±SE) temperature was 28°C (± 0.12) with mean (±SE) daily minimum and maximum temperatures of 26.1°C (± 0.17) and 31.5°C (± 0.14), respectively. One wing each from a random selection of emerged females was measured from the tip (excluding fringe scales) to the axillary incision in order to see if the neem affected adult mosquito size.

3.3.5 High Performance Liquid Chromatography (HPLC)

HPLC analysis was carried out to demonstrate the solubilisation of the compounds, to quantify certain constituents against known standards and to see if the amount of these (both known and unknown) varied with concentration of the aqueous neem extract. Extracts (five replicate samples each) representing four concentrations of the aqueous neem used in the bioassays were analyzed. They were made during the same serial dilutions but were not used in the bioassays. These samples were filtered, lyophilized (freeze dried) and dissolved in 1 ml

methanol. Fifty micro litres of this was then analyzed by HPLC. Analytical HPLC was performed on a Beckman System Gold Programmable Model 126 (Beckman Coulter International, Griesheim, Germany), using a Beckman reverse phase C18 column (5 µm x 4.6 mm x 25 cm) and eluted isocratically with acetonitrile and water (40:60). The flow rate was 1 ml/min and eluting constituents were monitored by a diode array detector module 168 at 214 nm.

To isolate standards (azadirachtin (AZA), nimbin, and salannin) with which to identify and quantify some of the peaks of the HPLC profiles, 5 g of neem seed cake powder (NCP) was suspended in 100 ml methanol at room temperature and stirred overnight. It was then filtered and evaporated to dryness in a rotary evaporator at 40^{o}C at reduced pressure (~337 mmHg). The residue was suspended in 100 ml water and extracted twice with 100 ml chloroform; the aqueous portions were discarded. The chloroform extracts were pooled and concentrated to dryness, again in a rotary evaporator at 40^{o}C under reduced pressure. The crude chloroform residue was then fractionated in a silica gel column using a hexane/ethyl acetate gradient. Column chromatography was performed on silica gel 60 (0.040-0.065 mm, 230-440 Mesh ASTM) and the fractions monitored by thin layer chromatography on a pre-coated silica gel 60 F_{254} plate (0.2 mm thickness) (Merck, Dermstadt, Germany). The ethyl acetate fractions contained the limonoids (including AZA, nimbin and salannin). Mass Spectrometry confirmed this.

3.3.6 Statistical analysis

3.3.6.1 Experiment one

As explained in **section 3.3.3,** the bioassay data from experiment one was analysed using probit analysis in SAS 9.1 (SAS Institute Inc. 2004) to enable the concentrations to be used in experiment two to be determined (data not shown). In addition, the development time of the mosquitoes was measured as the mean emergence time for the adults for each concentration. After determining equal or

unequal variance, two-sample t-tests were used to analyze the difference in development times between the control and neem treatments. For pupae, single factor ANOVA was used. Where a significant effect was seen, the Tukey multiple comparison test was carried out. Pupal mortality was analyzed using chi-square tests. Where the abnormalities were found in both the neem-exposed and control mosquitoes, they were analyzed using the Fishers exact test due to the low frequency of the abnormalities in the controls.

3.3.6.2 Experiment two

Due to the fact that the experiment two bioassay data didn't fit either the probit or logit models, we used the Weibull model of percentage mortality against the aqueous neem extract concentration. To avoid overflow of logarithm operation, we used Weibull transformation of $Y=LnLn\,(100.1/\,(100.1-P))$ on percentage mortality, P, and Log-transformation of $X=Ln\,(C+0.0001)$ on aqueous neem extract concentration, C. The transformation of P and C yielded a linear relationship $Y=a+bX$ and we subjected this to linear regression analysis (Seber and Wild 1989, Collett 2003). The IE_{50} and IE_{90} were computed via inverse prediction of linear regression and the 95% confidence interval (95% CI) was obtained verses inverse transformation. All data transformation and analysis was done using SPSS 14.0 (SPSS Inc 2005). In addition, the sex ratio of emerged mosquitoes was analyzed using the chi-square test. Pupal mortality was also analyzed using chi-square tests. Single factor ANOVA analysis was used to investigate the effect of the neem concentrations on the size of the emerged adult females. Statistical analyses were carried out at the 5% significance level.

3.4 Results

3.4.1 Experiment one

3.4.1.1 Development time

Apart from one LIV in the 1 g/l concentration, only the 0.1 g/l neem allowed emergence of adults when larvae were exposed. When compared to the control development time for each larval instar, there were significant increases in development time for LI, LII and LIII instars exposed to 0.1 g/l (Table 3.1). For pupae there was a significant effect of the treatment on the development time of the mosquitoes ($F=3.19$, $df=5,55$, $P<0.05$). Tukey multiple comparison analysis showed that the development time of Bti-exposed mosquitoes was significantly longer than for those exposed to 100 g/l neem (attributable to the fast mortality of pupae at this neem concentration with few adults emerging), but not significantly different from any other treatments (data not shown).

Table 3.1. Mean ±SE development times (in hours) of *An. gambiae s.s.* larval instars successfully emerging as adults after being exposed to 0.1 g/l neem extract or the control

Instar	Con. (N)	Neem (N)	Control	Neem	t-stat	df	p
LI	59	23	171.7 ± 2.3	218.1 ± 4.0	10.44	80	<0.0001
LII	69	34	146.1 ± 2.0	175.8 ± 2.4	9.05	101	<0.0001
LIII	67	28	120.4 ± 2.1	146.6 ± 3.1	6.93	93	<0.0001
LIV	85	64	97.4 ± 1.6	94.5 ± 2.1	1.11	147	=0.27

Con. = Control

3.4.1.2 Inhibition of pupation

At the neem concentration of 1 g/l larvae exposed as LI produced no pupae. For LII and LIII, two and fourteen dead pupae respectively were found; no live pupae were seen at any checkpoints. For LIV some live pupae were found, but only 0.8% (1/124) of the LIV exposed to 1 g/l emerged successfully as an adult. At 10 g/l larvae exposed as LI, LII, LIII, or LIV produced no live or dead pupae.

3.4.1.3 Pupal mortality

Significant mortality was seen at 10 g/l (χ^2=45.93, df=1, p<0.0001) but not at 1 g/l (χ^2=0.22, df=1, p=0.64) when compared to the control.

3.4.1.4 Abnormalities seen

A number of abnormalities were seen in the dead mosquitoes, albeit at low frequencies. Of these, three types occurred in only the neem-exposed mosquitoes. The most frequently occurring of these, seen in Figure 3.1, was only found in pupae (21 of the 500 exposed) and was similar to that reported by Vasuki and Rajavel (1992). These pupae all had abnormally large amounts of fluid in their abdomens. Also their whole bodies were outside of the pupal case. However, it did not look like they were trying to emerge as the head, thorax and abdomen were tucked together in a straight line, with the legs close in to the abdomen (Figure 3.1). The second most frequent abnormality was a dark band across the thorax or abdomen occurring in 49 of the 1,999 neem-exposed larvae. Finally, we found tracheal tube coloration in seven dead larvae. There were three other abnormalities that also affected some control mosquitoes. The most frequently occurring was what looked like rectal prolapse as described by Raghunatha Rao et al. (1988). Significantly more neem-exposed larvae (25/1,999) had this abnormality when compared to the controls (1/497) (p<0.05). The other two abnormalities were black anal papillae (1/497 control; 18/1,999 neem-exposed; p=0.33) and exuviate still

attached (3/497 controls; 17/1,999 neem-exposed; p=0.78), the latter being previously described as a neem-induced abnormality (Raghunatha Rao et al. 1988).

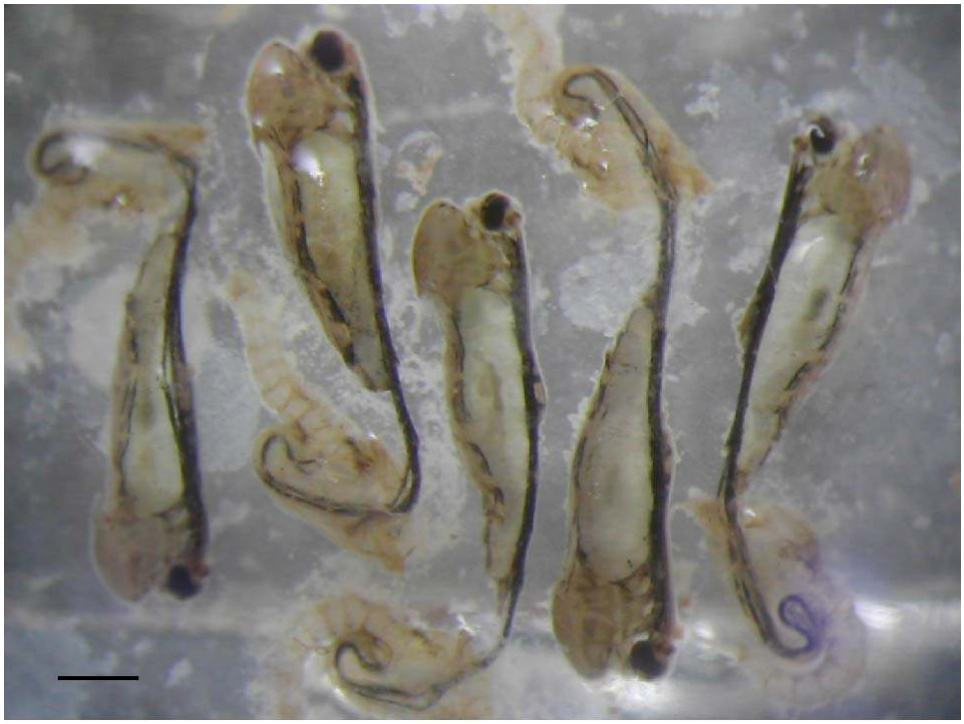

Figure 3.1. Neem-induced abnormality causing death in pupae; the scale bar represents 1 mm

3.4.2 Experiment two

3.4.2.1 Bioassay data analysis

The linear regressions on the transformed data yielded very significant results, implying that the data fitted the Weibull model well. The slope (on the Log-scale) of the regression equation was greatest for LI larvae and least for pupae. Similarly, the IE_{50} and IE_{90} values were lower for LI and highest for pupae (Table 3.2).

Table 3.2. IE_{50}, IE_{90} results (g/l) and other related parameters from the analyses (using the Weibull model) of the mortality obtained from exposing different stages of *An. gambiae s.s.* to a range of neem concentrations in aqueous extracts from wood and bark chippings

Life stage	**N**	**Adjusted R^2**	**df**	**F**	**p**	**Slope (95% CI)**	**IE_{50} (95% CI)**	**IE_{90} (95% CI)**
LI	625	0.66	1, 18	37.34	<0.0001	1.11 (0.73, 1.49)	0.07 (0.01, 0.47)	0.12 (0.02, 0.80)
LII	625	0.81	1, 23	107.67	<0.0001	0.81 (0.65, 0.98)	0.11 (0.02, 0.58)	0.15 (0.03, 0.79)
LIII	625	0.87	1, 23	158.54	<0.0001	0.94 (0.79, 1.10)	0.18 (0.05, 0.68)	0.14 (0.04, 0.56)
LIV	625	0.78	1, 23	94.08	<0.0001	0.80 (0.63, 0.97)	0.40 (0.05, 3.27)	0.63 (0.07, 5.35)
Pupae	625	0.89	1, 23	189.95	<0.0001	0.27 (0.23, 0.31)	58.16 (3.97, 850.98)	61.57 (3.8,989.8)

3.4.2.2 Emerged sex ratio

There were no significant differences between the sex ratios of emerged adults of the neem-exposed and control mosquitoes for any of the larval instars tested (data not shown). For pupae there was a small significant difference (χ^2=11.5, df=4, p<0.05) with the controls producing more females.

3.4.2.3 Elongation of larval development time

For all larval instars at a range of neem concentrations the LIV instar stage was prolonged for an unusually long time in some individuals (Table 3.3). The affected larvae were unable to moult into pupae and died as LIVs.

3.4.2.4 Inhibition of pupation

Just 0.8% (1/125) of the LI exposed to 0.2 g/l led to successful adult emergence. Pupation was completely prevented at 0.8 g/l for LI. For LII larvae some dead pupae were found at 0.8 g/l. Only one LIII larva produced a live pupa at 0.8 g/l which died the next day. At 1.6 g/l no live pupae were produced from LIV larvae, however, 39% (49/125) of the larvae exposed as LIVs died as pupae at this concentration.

3.4.2.5 Pupal mortality

The control mortality when using distilled water was 42% (106/250 died). The five replicates with the purified water 'Dasani ™' produced only 8/125 (6%) control mortality. When using the 'Dasani ™' water, significant pupal mortality was seen at 5 g/l aqueous neem extract (χ^2=53.6, df=1, p<0.0001), the lowest concentration tested.

Table 3.3. Number (N) and larval stage where developmental arrest and subsequent mortality took place at different concentrations of neem extracts

Larval stage	Neem (g/l)	Arrested and died as	N	Min. time in that instar (days)	Day of death
LI	0.2	LIV	4	6 – 9	14 – 15
LII	0.8	LIII	1	6	10
LII	0.8	LIV	1	6	11
LIII	0.2	LIV	1	7	9
LIII	0.8	LIV	5	7 – 9	9 – 11
LIII	1	LIV	1	8	12
LIII	10	LIV	1	7	9
LIV	1	LIV	2	8	8
LIV	1.6	LIV	3	9	9

Table 3.4. HPLC results showing mean ± SE quantities (in mg/g) of nimbin and salannin in aqueous neem extracts of different concentrations

Neem (g/l)	N	Nimbin (mg/g)	Salannin (mg/g)
0.0125	5	*a*	*a*
0.2	5	*a*	*a*
5	5	0.0095 ± 0.006	0.0071 ± 0.003
180	5	0.045±0.02	0.014±0.004

a = the amounts present were too small for quantification

3.4.2.6 Wing lengths

Despite the larvae in the higher neem concentrations being visually smaller in size than those in the controls, there was no significant reflection of this effect on the wing length (and therefore their body size (Briegel 1990)) of the emerged adult females for any of the instars tested (n = 276; data not shown).

3.4.2.7 HPLC

Nimbin and salannin were found in our aqueous extracts. The peak that had the same retention time value as AZA was isolated by repeated semi-preparative HPLC runs. The pooled sample was then examined by Mass Spectroscopy and compared with AZA isolated from NCP (solid probe spectrum). However, it did not correspond to that of AZA isolated from NCP. Thus, AZA was not present in our samples in significant amounts. In addition to the less polar nimbin and salannin, HPLC profiles of the aqueous extracts showed a series of more polar compounds had dissolved. For the highest concentration tested (180 g/l) there were up to a minimum of 20 distinct peaks (Figure 3.2). The profiles for each of the five samples at the different concentrations look similar indicating that for each batch of water made, a similar group of neem wood constituents had leached out into the water, and for each concentration, in comparable quantities. By comparison with authentic samples of nimbin and salannin, we were able to quantify the level of these compounds (in mg of compound per gram of sample) in our profiles obtained from higher concentrations (Table 3.4). However, for the two lowest concentrations no distinct peaks corresponding to these compounds could be discerned at the concentrations analyzed and therefore no quantification was possible.

3.5 Discussion

Our results show that the IE_{90} for LI, LII and LIII *An. gambiae s.s.* is around 0.15 grams of neem wood per litre water; for LIV it is 0.63 g/l. The higher susceptibility

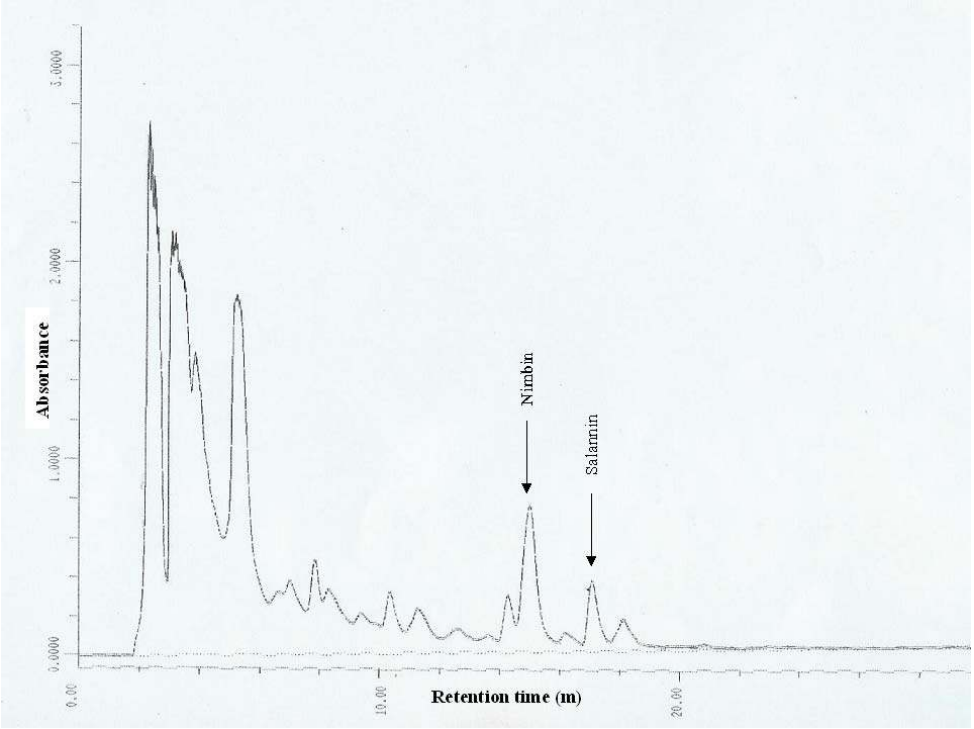

Figure 3.2. High Performance Liquid Chromatography (HPLC) profile of 180 g/l crude aqueous neem wood extract

of the younger larval instars is in line with previous work (Mulla and Su 1999, Nathan et al. 2005). However, the exposure time for the younger instars was longer. Also, the overlapping confidence intervals in our data show that there is no significant difference between the larval instars exposed to neem with respect to their susceptibility to the neem extract. The IE_{90} for pupae, on the other hand, was much higher at 61 g/l and significant mortality was seen at 5 g/l. Despite this, even for 180 g/l the highest mortality seen was 88%; there was always some emergence. Aqueous extracts of neem wood chippings at relatively low doses could therefore be used to control larval populations of *An. gambiae s.s.* but at the doses used for this, we would not expect pupae to be significantly affected.

We observed, but did not specifically study or quantify, several behavioural and physiological effects of the neem that could make the larvae more susceptible to natural predators in the wild. Firstly, the larvae exposed to 1 g/l neem and above were less responsive to visual and mechanical stimuli. Secondly, the neem-exposed larvae seemed to move sluggishly as previously reported (Nathan et al. 2005) and were more likely to spend time at the surface of the water and in the middle of the bowls rather than trying to hide at the bottom or at the sides. Finally, the increase in development time would mean they would be exposed to predators for longer periods, thus increasing their chances of being predated (Tuno et al. 2005).

Limonoids from neem seed kernels have well known antifeedant effects against different insects (Schmutterer 1995), however, this mode of action is difficult to assess in aquatic organisms. We noticed that larvae in the higher neem doses were smaller than the controls, indicating that they were consuming fewer nutrients; we did not measure this. However, we found no significant effect of the neem on the size of emerged females. Thus, if the phytochemical blend of neem wood chippings was working as an antifeedant then the effect was slight. Both the elongation of the larval stage and inhibition of pupation observed in this study have been reported previously (Mulla and Su 1999, Ndung'u et al. 2004, Okumu et al. 2007). These effects could have been because the mosquitoes had not attained the critical mass needed for pupation (Clements 1992). Alternatively, disruption of their normal development by the growth-disrupting effects of neem limonoids may be the underlying factor.

HPLC analyses of our aqueous extracts showed the presence of constituents of different polarity including the relatively less polar nimbin and salannin. Interestingly, nimbin does not usually dissolve significantly in water but was present in prominent amounts in our samples. The presence of other constituents may promote the solubility of the less polar constituents in the aqueous medium to provide a blend of different compounds. A major benefit of crude raw materials

rather than extracts with limited profiles of constituents is the possibility of synergistic or additive effects between some of these compounds (Isman et al. 1996, Bekele and Hassanali 2001, Ndung'u et al. 2004). Crude extracts of whole plant tissue are also easier to use in resource-poor rural settings.

There are many active compounds in neem (Schmutterer 1995, Mulla and Su 1999) with up to 100 different limonoids (Isman et al. 1996). Therefore, since we did not detect AZA in our samples the inhibition of larval growth and metamorphosis observed must be due to other compounds. Both salannin and nimbin have been found to inhibit ecdysone 20-monooxygenase (an enzyme important for moulting) (Mitchell et al. 1997), albeit with much less effect than AZA. In addition, the absence of AZA may explain why our IE_{50}s were slightly higher than those found in other studies (Mulla and Su 1999, Nathan et al. 2005, Okumu et al. 2007).

Several other studies have shown the effectiveness of neem-based pesticides against mosquito larvae. However, the only previous work with neem wood/bark and *An. gambiae* was carried out by Ziba (1995) on LIII and LIV larvae. Ziba tested the leaves, bark and seeds and assumed by association that AZA was the primary active agent and present in all the parts of the tree. No analyses of the extracts were carried out to demonstrate if AZA was present. Ziba's results showed that the bark and leaves caused 87% mortality of *An. gambiae* larvae after 24 hours exposure to 1:10 dilutions of 100% w/v (Ziba 1995). For LIII we also found 87% mortality after 24 hours for 100 g/l; for LIV it was 59%.

A frequent concern is how neem products will affect humans if placed into their water sources. A review on the toxicological effects of neem-based products on mammals, including humans, showed that the aqueous extracts were less toxic than other neem extracts, and they quoted an acceptable daily intake of aqueous neem leaf extract as 0.3 mg/kg body weight/day. These authors concluded that "if applied with care the use of … aqueous neem-based products should not be discouraged" (Boeke et al. 2004b). A separate study also concluded that "no

ecological hazard is likely to result" from the use of the neem-based pesticides (Goktepe et al. 2004).

What sets this work apart from other work involving phytochemicals against mosquito larvae is that although other more sophisticated extracts may produce encouraging results (Nathan et al. 2005), they are impractical to produce and therefore be used by the resource-poor people in rural Africa. The aqueous extract is more applicable to rural situations where malaria causes the greatest burden. The neem tree is already well known and grows all over Africa, excluded only by altitudes of over 1,000 m (Schmutterer 1995). At these high altitudes, other Meliaceae species such as the shrub *Turraea mombassana* Hiern ex. C.DC. could be used (Ndung'u et al. 2004). In addition, neem would be much more affordable than other larvicides (e.g. Bti which is far too expensive), especially if grown in the country needed rather than imported, therefore also providing local income.

Other benefits of neem are that it is biodegradable, relatively safe to the environment and easy to apply. The blend of active and synergistic compounds found in neem means that it is less likely that the mosquitoes will build up physiological resistance (Isman et al. 1996). Awad and Shimaila (2003) used the selective pressure of neem oil for five generations and found no change in the susceptibility of anopheline mosquitoes. They also found no resistance in the field after using it biweekly for three months. Therefore, neem chippings could be used as an additional component of a rotational IVM strategy to reduce resistance in the target population. Also, neem used as a larvicide has been shown to reduce adult mosquito longevity (Nathan et al. 2005, Okumu et al. 2007).

The focus of the present study on need wood chippings rather than leaves may raise questions. This is because the leaves are easier to harvest, especially at an earlier stage of the tree development, and they are more able to regenerate. On the other hand, against *An. gambiae* the leaves and bark have been found to be equally effective (Ziba 1995). Also, compared to the wood/bark there has already been extensive work done on the leaves. We used the wood of the tree as neem

trees react well to pruning, re-growing quickly (Office of International Affairs 1992) making wood chippings continuously available for this purpose. We also expect that it would take more time for the active ingredients to leach from the wood (when compared to the leaves), thus providing a controlled-release mechanism for delivering active ingredients into treated anopheline pools and prolonging the effect of the insecticide.

In summation, in the present study we evaluated aqueous extracts of the wood and bark of neem trees and showed that it has the potential to be a good source of control agents for *An. gambiae s.s.* larvae. Aqueous neem extract also has the potential to be a low-tech control method that could be produced in Africa and integrated into vector control schemes. Neem is locally available in Africa, it is biodegradable and its application is relatively simple.

3.6 Acknowledgements

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Chapter 4

Effects of a botanical larvicide derived from *Azadirachta indica* (the neem tree) on oviposition behaviour in *Anopheles gambiae s.s.* mosquitoes

Annabel F. V. Howard, Elizabeth Adongo,

John Vulule, John Githure

Submitted

4.1 Abstract

Mosquito larval control is being given more focus due to adult insecticide resistance and the necessity to use several control techniques together in integrated vector management (IVM) programmes. Botanical products are thought to be able to provide effective, sustainable and cheap mosquito larval control tools. However, bio-larvicides like *Azadirachta indica* (neem) have been found to repel adult mosquitoes from laying their eggs in the treated larval habitats. A larval control tool cannot be sustainable and effective at controlling successive generations if it prevents female mosquitoes from exposing their progeny to the control tool, especially when non-treated oviposition sites are available. In the laboratory we examined the response of *Anopheles gambiae s.s.* mosquitoes towards varying doses of crude aqueous neem extracts. This simple extract was used because it is more likely to provide sustainable control in the field than refined extracts requiring complex equipment and infrastructure. We used non-choice oviposition tests to measure the proportion of mosquitoes laying on the first or second night, or not laying at all, when compared to the control. For each individual mosquito, the number of eggs laid and/or retained in the ovary was counted to determine the relationship between wing length and egg production. The results show that larger females produced larger egg batches. We also found that at a dose of 0.1 gram of neem wood in 1 litre of water, a concentration we have previously found to be effective at controlling mosquito larvae, significantly more mosquitoes laid eggs when compared to the control. Previous work has also found that low doses of mosquito repellents can actually attract mosquitoes. At the higher doses no significant repellency was found. The finding that raw neem does not repel mosquitoes from laying their eggs when used in a simple application method is of interest because the premise of IVM programmes is that communities will be more involved in mosquito control. As such, simple application methods of raw botanical products that require almost no infrastructure are likely to be given more prominence. At all doses tested, our results indicate that the mosquitoes will

expose successive generations to the control tool, making the use of simple aqueous neem extracts effective and potentially sustainable. Field trials should be carried out to monitor whether the behavioural response of wild mosquitoes will be similar.

4.2 Introduction

Malaria is arguably the most important tropical parasitic disease in the world. Transmission is centred on the tropics and globally it is estimated that half of the world's population is at risk (Hay et al. 2004, World Health Organisation 2009). Almost one million people were estimated to have died from malaria in 2008, and there were over 240 million cases (World Health Organisation 2009). Human malaria is transmitted by female *Anopheles* spp. (Diptera: Culicidae) mosquitoes when they take a blood meal.

The process of taking a blood meal, egg maturation and oviposition (egg laying) in mosquitoes is called the gonotrophic cycle (GC); females can have multiple GCs in their lifetime. Once eggs are mature, *Anopheles gambiae* Giles mosquitoes have a peak of flight activity at dusk, thought to be associated with oviposition-site selection (Jones and Gubbins 1978); oviposition itself occurs at night (McCrae 1983) over a two-to-four hour period (Fritz et al. 2008). In the field, *An. gambiae* are exposed to various different biotic and abiotic factors in natural (Gimnig et al. 2001, Minakawa et al. 2004) and man-made habitats (Mutuku et al. 2006b, Howard and Omlin 2008) (**Chapter 5**). Ovipositing mosquitoes can discriminate between these different biotic and abiotic factors using visual, semiochemical and physicochemical cues (Takken and Knols 1999). Mosquito larvae (McCrae 1984, Munga et al. 2006), competitors (Munga et al. 2006), predators (Angelon and Petranka 2002, Blaustein et al. 2005), botanical extracts (Dhar et al. 1996, Elimam et al. 2009) and some types of bacteria (Huang et al. 2006) can repel mosquitoes from ovipositing, whilst other types of bacteria (Lindh et al. 2008), fungi

(Sivagnaname et al. 2001) and low levels of conspecific larvae (Sumba et al. 2008) can attract ovipositing mosquitoes.

Due to widespread insecticide resistance in adult mosquitoes (Hemingway and Ranson 2000), attention has been refocused towards the pre-insecticide era control tools including larval control and environmental management (Killeen et al. 2002, World Health Organisation 2009). These methods are getting more focus because the World Health Organisation recommends that malaria be tackled using integrated vector management (IVM) which uses all available control techniques that are locally appropriate and sustainable (World Health Organisation 2004b). Non-chemical larval control can either use natural predators (Ghosh and Dash 2007, Howard et al. 2007) (**Chapter 6**), entomopathogenic fungi (Bukhari et al. 2010) or botanical larvicides (Shaalan et al. 2005). However, for whichever method is to be used, it is important to determine whether mosquitoes will continue to oviposit in treated larval habitats. This is because if a treatment repels mosquitoes from ovipositing then it will not be able to control successive generations of mosquitoes; if a treatment does not repel mosquitoes then the females will still expose their progeny to the larval control tool.

One botanical larvicide that has received much attention recently is derived from *Azadirachta indica* A. Juss (Meliaceae) (the neem tree). Extracts of different parts of this tree have been effective at killing mosquito larvae both in the laboratory (Okumu et al. 2007, Howard et al. 2009) (**Chapter 3**) and field (Awad and Shimaila 2003, Gianotti et al. 2008). Furthermore, this tree grows in many African countries and could potentially be a sustainable component of IVM programmes (**Chapter 2**). However, in the laboratory neem has been found to be an oviposition deterrent for mosquitoes (Dhar et al. 1996). A study with *Anopheles stephensi* Liston and *Anopheles culicifacies* Giles using a range of neem extracts found that 7 day old gravid mosquitoes exposed to neem volatiles for 90 minutes exhibited oviposition suppression, with neem-exposed females retaining significantly more eggs than control mosquitoes (Dhar et al. 1996). Females that were exposed to neem-derived

volatiles immediately after mating and were left exposed to these volatiles for several days did not fully develop eggs either in that or successive GCs (Dhar et al. 1996). Similarly, when neem was fed to *An. stephensi* mosquitoes either before or during a blood meal, egg maturation and oviposition were adversely affected (Lucantoni et al. 2006).

Previously we have shown that a dose equivalent to 0.1 grams of neem wood per litre of water causes a significant increase in larval *An. gambiae* Giles *s.s.* development time, and was also able to cause significant levels of mortality (Howard et al. 2009) (**Chapter 3**). In this study, we used *An. gambiae s.s.* mosquitoes in non-choice experiments to test whether the 0.1 g/l and other doses of crude aqueous extracts of *A. indica* (neem) affected mosquito oviposition behaviour.

4.3 Materials and Methods

4.3.1 Preparation of aqueous insecticidal extracts

Neem extracts were prepared as previously described (Howard et al. 2009) (**Chapter 3**). Briefly, wood and bark from neem trees collected from Mbita Point in western Kenya were fed into a basic wood chipping machine to produce wood chippings (roughly 1 x 3 x 0.2 cm), which were left to dry in the shade. These dry chippings were then soaked in distilled water for five days after which time the water was filtered, removing the neem chippings and leaving just the aqueous extract into which the neem phytochemicals had leached. This simple method was used because it is more likely to provide sustainable control in the field than refined extracts requiring complex equipment and infrastructure. The different concentrations used in the oviposition experiments (equivalent to 0.1, 1 and 10 grams neem wood per litre water) were made by serial dilution from a stock solution. Distilled water was used for the controls.

4.3.2 Mosquitoes

The Kisumu strain of *An. gambiae s.s.* was used. This strain has been maintained as a colony at the Kenya Medical Research Institute (KEMRI), Kisumu, for 17 years. After standard rearing, pupae were separated and placed into an adult cage for emergence. The following day any live pupae that had not emerged during the night were removed from the cage to ensure all adults were the same age. Both male and female adults were kept in the cage to allow mating to occur. *Anopheles gambiae s.s.* host seeking peaks at day 4 post emergence (Takken et al. 1998), so once adults were four days old, females were blood fed on a live rabbit for 30 minutes. One hour after feeding, female mosquitoes that had ingested a full blood meal were moved to a new cage along with a number of male mosquitoes to allow unmated females to mate. Mating can increase the chance of egg maturation (Klowden and Russell 2004) and females mating after a blood meal are as likely to oviposit as those that mate before a blood meal (Chambers and Klowden 2001).

Two days after the first blood feed, female mosquitoes were again allowed to feed from a live rabbit because sometimes anophelines require multiple blood meals to develop their first batch of eggs (Clements 1992, Briegel and Horler 1993, Takken et al. 1998) and host seeking is still peaking at day 6 post emergence (Takken et al. 1998). One hour after this second feed, females that had blood fed or that were already semi-gravid from the first feed were further separated into another cage. Males were also placed into the new cage. These mosquitoes were left for a further three days before the females were used in the oviposition experiments. Although leaving mosquitoes that had first fed five days previously without an oviposition site may seem a long time, previous research has shown that retention of mature eggs by *An. gambiae* females until an oviposition site is available does not adversely affect oviposition (Chambers and Klowden 2001).

Throughout this whole process mosquitoes had access to 10% sugar solution soaked in cotton wool that was placed onto the roof of the cage and refreshed daily.

4.3.3 Oviposition experiment

Non-choice experiments were carried out to investigate the effect of the water treatment on whether mosquitoes chose to lay their eggs and if so, if the mosquito laid at the first opportunity or waited until it became obvious no other option was available. Standard (30 x 30 x 30 cm) wire frame cages covered in cotton netting were used for the experiments. The wooden bottoms of these cages were painted black because more *An. gambiae s.l.* eggs are laid over dark than light areas (McCrae 1984). For the oviposition sites, 40 ml of neem-treated or control water was soaked onto cotton wool in a Petri dish. A 90 mm filter paper was then placed on top of this wet cotton wool. At 5pm a single gravid female mosquito and one Petri dish were randomly allocated to each cage. Cotton wool soaked in 10% sugar solution was placed onto the roof of each cage and refreshed daily. The mosquitoes were exposed to a natural dusk and left in a natural 12:12 hr L:D cycle. The mean (±SE) maximum and minimum temperatures during the study were 30ºC (±0.10) and 25ºC (±0.11) respectively; the mean (±SE) humidity was 80% RH (±0.11).

The next morning, any mosquitoes that had died or were stuck to the filter paper were removed from the experiment. For mosquitoes continuing with the experiment, Petri dishes were removed from the cages and the number of eggs on each was counted using a dissection microscope. The Petri dishes were then put back into the cages. Mosquitoes were left in the cage to allow them to oviposit during the second night of the experiment. The following morning any mosquitoes that had died or were stuck to the filter paper were removed from the experiment. The Petri dishes were removed and the number of eggs counted again using a dissection microscope. Mosquitoes were removed for dissections as described in **section 4.3.4** below. Thirty replicates were carried out per water treatment (not including mosquitoes failing to complete the experiment).

4.3.4 Mosquito dissections

The morning after the second experimental night, mosquitoes were individually removed from the cages, knocked down in the freezer for 5-10 minutes and then dissected. Dissections were carried out on glass slides using hypodermic needles under a dissection microscope. Firstly, a dry dissection was carried out and one wing was randomly selected and removed from each mosquito and placed on a separate glass slide. Wings were measured from the tip (excluding fringe scales) to the axillary incision using a compound microscope and ocular micrometer.

For the wet ovary dissections, 0.85 g AnalaR salt (NaCl) was put into 100 ml distilled water to make a saline solution. A few drops of this saline solution were used to aid mosquito ovary removal. Ovaries were then gently opened and the number of eggs remaining inside was counted using a dissection microscope.

4.3.5 Statistical analysis

Although all mosquitoes had taken at least one blood meal, a previous study has found that around 15% of blood fed *An. gambiae s.s.* do not mature eggs (Hogg 1996). Therefore, as well as the 9.7% (14/144) mosquitoes that died or stuck to the filter paper, the 5.5% (8/144) mosquitoes that had not developed eggs were also discarded from both types of analyses.

4.3.5.1 Effect of neem on oviposition

The purpose of this study was to see if neem treatments would cause the mosquitoes to retain their eggs either for oviposition on the second night or in their ovaries at the end of the experiment. Therefore, we were interested in looking at whether the treatments had caused the number of mosquitoes that laid/retained their eggs to vary, rather than examine the number of eggs laid in each treatment. To analyse whether the number of mosquitoes laying or retaining eggs significantly

differed between the four water treatments, we coded the mosquitoes as having laid (1st or 2nd night) or retained eggs, and analysed the coded data using chi-square tests.

4.3.5.2 The relationship between wing length and egg production

After testing to see if the data sets (wing lengths and total number of eggs produced (laid plus retained) per mosquito) were normally distributed, single factor ANOVA was used to test for any significant differences in the number of eggs produced by females exposed to each treatment. Similarly, single factor ANOVA was used to test for any differences in the wing length of females exposed to each treatment. No significant differences were found so the data were pooled and the correlation between wing length and number of eggs produced was analysed using simple linear regression. To see if there was a significant difference between the numbers of eggs that small and large mosquitoes produced, a two-sample t-test assuming equal variances was carried out. Analyses were carried out in SPSS 17.0 (SPSS Inc 2008) with α set at 0.05.

4.4 Results and Discussion

4.4.1 Effect of neem on oviposition

There were no significant differences in the total number of eggs produced (laid plus retained) ($F=2.39$, $df=3,118$, $p=0.07$) between mosquitoes exposed to the four treatment types, indicating that when fully gravid mosquitoes are exposed to neem, the exposure does not significantly affect egg production (i.e. by making mosquitoes reabsorb eggs (Clements 1992)).

We found that mosquitoes either laid all of their eggs on one night, or retained all of

their eggs. Only 4.1% (5/122) of mosquitoes laid their eggs over a number of nights, and these were distributed between the four treatment groups. In addition, most of the mosquitoes that were going to lay their eggs did so on the first night (Figure 4.1), just 10.7% (13/122) of the mosquitoes laid their eggs on the second night. Of these, only one was exposed to the control treatment and four mosquitoes came from each of the neem treatments. However, there was no significant difference between the four water treatments with respect to the day mosquitoes laid their eggs (χ^2=1.1, df=3, p=0.77).

Sixty percent of the mosquitoes exposed to control water laid their eggs on the first night, and a further 6.7% laid on the second night. This left 33.3% of the mosquitoes with eggs retained in their ovaries (Figure 4.1). Chambers and Klowden (2001) had a similar finding with just two-thirds of their *An. gambiae s.s.* females ovipositing their eggs in two consecutive nights. For the lowest neem concentration of 0.1 g/l, 75.8% of the mosquitoes laid eggs on the first night, a further 13.8% laid on the second night and just 10.4% of mosquitoes retained eggs in the ovaries (Figure 4.1). When comparing mosquitoes that laid eggs with those that retained their eggs, significantly more mosquitoes laid their eggs when exposed to the low neem dose of 0.1 g/l when compared to the control-exposed mosquitoes (χ^2=4.5, df=1, p=0.033). For both the 1 g/l (χ^2=0.0, df=1, p=1.0) and 10 g/l (χ^2=0.5, df=1, p=0.458) there were no significant differences in the number of mosquitoes either laying or retaining their eggs when compared to the control mosquitoes. This lack of repellent effect in the higher doses may be due to the lack of detectable azadirachtin in these crude aqueous neem extracts (Howard et al. 2009) (**Chapter 3**).

The fact that more eggs were laid in the 0.1 g/l might be explained by two mechanisms. Firstly, previous work comparing *An. gambiae s.l.* oviposition in distilled and natural field water has found that more eggs are laid in the natural water (McCrae 1984, Sumba et al. 2004, Sumba et al. 2008) because this water contains bacteria. We appreciate that using such a simple neem phytochemical

extraction method would allow some bacteria to be in the extracts because our neem wood was not sterile before being soaked in water. The second possible explanation is that low doses of aqueous neem wood extract act as oviposition attractants for *An. gambiae s.s.* mosquitoes. In agreement with this, previous findings have shown that low doses of mosquito repellents can actually act as attractants (Skinner et al. 1980, Mehr et al. 1990). These factors could individually be responsible, or there may be an element of interaction between the two.

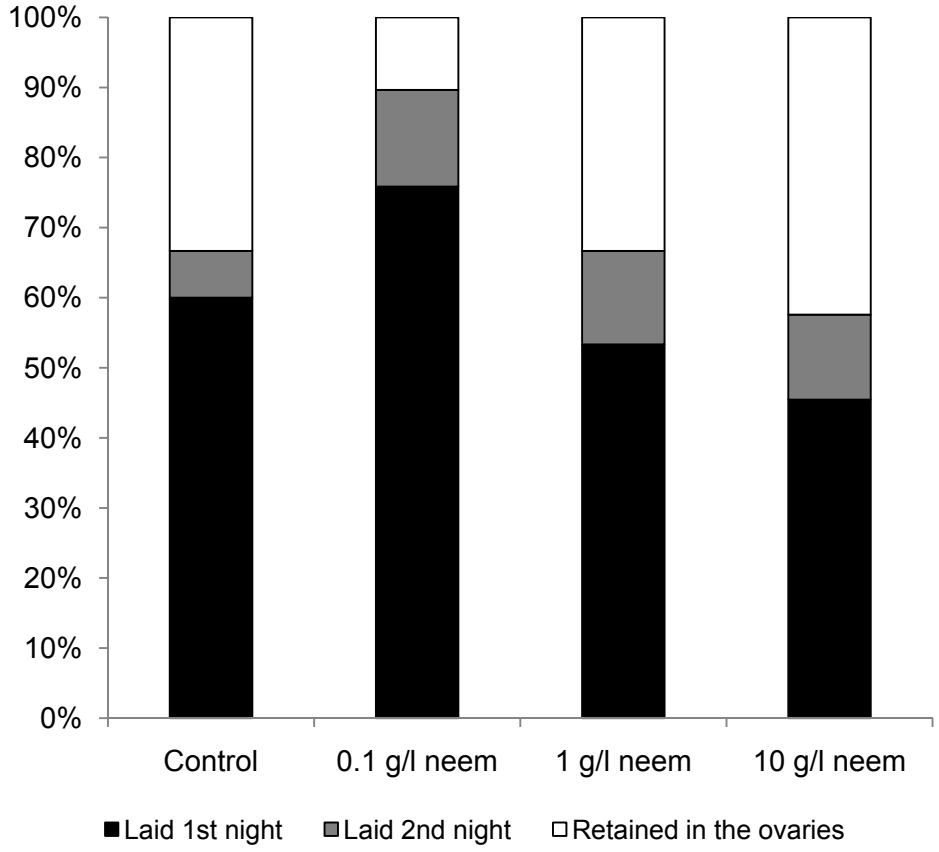

Figure 4.1. Proportional breakdown of mosquitoes laying eggs on the first (black) or second (grey) nights, and those retaining eggs in the ovaries (white) after being exposed to control water (N=30) or aqueous neem extracts at concentrations of 0.1 g/l (N=29), 1 g/l (N=30) or 10 g/l (N=33)

That the 0.1 g/l neem extract enhanced mosquito oviposition is encouraging. In a previous study we used the same type of neem-treated water and the same mosquito strain and found that at 0.1 g/l, larvae exposed during the first three instars had significantly increased development times when compared to larvae reared in control water (Howard et al. 2009) (**Chapter 3**). In addition, the concentration that inhibited 90% of adult emergence (IE_{90}) was around 0.15 g/l for early instar mosquito larvae (Howard et al. 2009) (**Chapter 3**). These oviposition results show that if neem wood was applied to water bodies at a concentration of around 0.1 g/l then not only would mosquito larvae take significantly longer to develop into adults, with significantly fewer surviving to adulthood (Howard et al. 2009) (**Chapter 3**), but the oviposition behaviour of the adult mosquitoes would be significantly enhanced, so successive generations of mosquitoes would keep being exposed to the botanical larvicide. In addition, ovipositing *An. gambiae s.s.* adults have been shown to exhibit a memory (Sumba et al. 2004) because they prefer to oviposit in the same water type in which they were reared, when compared to water in which another *An. gambiae s.s.* strain was reared (Ogbunugafor and Sumba 2008). This preference for "known" water has even been found when mosquito repellents were placed in water (Kaur et al. 2003); *Aedes aegypti* L. mosquitoes reared in water containing citronella and neem exhibited reduced repellence towards ovipositing in the treated water than mosquitoes reared in clean water (Kaur et al. 2003). Our results suggest that at 0.1 g/l, *An. gambiae s.s.* larvae can be considerably controlled (Howard et al. 2009) (**Chapter 3**) and females will still oviposit in the water. Given previous findings about mosquito memory (Kaur et al. 2003, Sumba et al. 2004, Ogbunugafor and Sumba 2008), any mosquitoes emerging from the neem-treated water may preferentially return to oviposit in that water, exposing their progeny to the control measure.

Previously, neem has been shown to repel mosquito oviposition. Dhar et al. (1996) used short exposures to show that gravid 7 day old *Anopheles* mosquitoes laid significantly more eggs in the control water when compared to mosquitoes exposed to broken neem seed kernels, purified neem oil and neem volatile fractions (Dhar et

al. 1996). Our results show no repellency caused by neem exposure and in fact the opposite was found for 0.1 g/l. It is promising that repellent properties were not found in our study when a simple application method was used. The expectation is that community involvement in mosquito control will increase as IVM programmes spread across Africa (World Health Organisation 2004b, van den Berg and Takken 2007) (**Chapter 2**). Communities are more likely to use mosquito control methods that require the least sophisticated equipment and infrastructure, and this will be especially true in resource-poor rural areas. Therefore, the finding that when raw neem wood is placed into water at a relatively low dose the proportion of mosquitoes ovipositing is enhanced is encouraging. In addition, no repellent effects were seen even at a dose 100 times that required for successful mosquito control (Howard et al. 2009) (**Chapter 3**). If this simple application of the control tool is to be used by rural communities, then the dose may not always be controlled. This could lead to overtly high doses being used, but our evidence suggests that even these very high doses will not adversely affect mosquito oviposition behaviour. However, these laboratory results need to be verified in the field, because it is possible that the oviposition response to neem is different in natural water bodies that produce a range of volatile signals.

4.4.2 The relationship between wing length and egg production

Whilst neem has been shown to affect egg development in mosquitoes when given before or with the blood meal (Lucantoni et al. 2006), the mosquitoes in our experiment had developed their eggs before being exposed to neem. As a result, we found no significant difference between the number of eggs produced by mosquitoes in the four treatment groups ($F=2.39$, $df=3,118$, $p=0.07$). In addition, there were no significant differences between the wing lengths of mosquitoes exposed to the four treatments ($F=0.05$, $df=3,118$, $p=0.98$), so the data were pooled for the purpose of examining the relationship between wing length and egg development.

The mean (±SE) wing length was 3.09 mm (±0.01), and the mean (±SE) number of eggs produced was 53.6 (±2.9). The number of eggs produced by individual mosquitoes was significantly (n=122; adjusted r^2=0.25; p<0.0001) and positively correlated in a linear fashion with wing length (Figure 4.2). Thus, 25% of the variation in the number of eggs produced is explained by the mosquito wing length. This positive correlation between wing length and the number of eggs produced has previously been found in laboratory colonies (Briegel 1990, Takken et al. 1998) and wild caught mosquitoes from Tanzania (Lyimo and Takken 1993), The Gambia (Hogg 1996) and Mali (Yaro et al. 2006).

We also found that providing two blood meals was sufficient to get even small mosquitoes to mature eggs. It has been previously suggested that *An. gambiae* females with wing lengths shorter than 3 mm are unable to start oogenesis after the first blood meal (Briegel 1990, Lyimo and Takken 1993). In our study, 27% (33/122) of mosquitoes that produced eggs had wings shorter than 3 mm (Figure 4.2).

Wing length is used as a measure of mosquito body size. Larger *An. gambiae* females have been shown to have higher levels of lipid, protein and carbohydrate at eclosion (Briegel 1990). They also take larger blood meals (Briegel 1990), are better able to utilize the meal (Takken et al. 1998) and are therefore able to produce more (Briegel 1990, Lyimo and Takken 1993, Hogg 1996, Takken et al. 1998) and larger (Takken et al. 1998) eggs. In addition, larger blood meals lead to a higher protein content per egg (Briegel 1990). Larger female mosquitoes therefore have a higher reproductive efficiency (fecundity) than smaller mosquitoes. In agreement with this, when mosquitoes from our study were categorised as being small (wing length <3.15 mm) or large (wing length ≥3.15 mm), there was a significant difference in the mean number of eggs that each group produced (t=6.26, df=120, p<0.0001) with small females producing a mean (±SE) of 40.7 (±2.9) eggs compared to 72.9 (±4.7) for large females. As well as producing more eggs, larger females also tend to live longer, host seek more

(Takken et al. 1998) and require fewer blood meals to become fully gravid (Lyimo and Takken 1993).

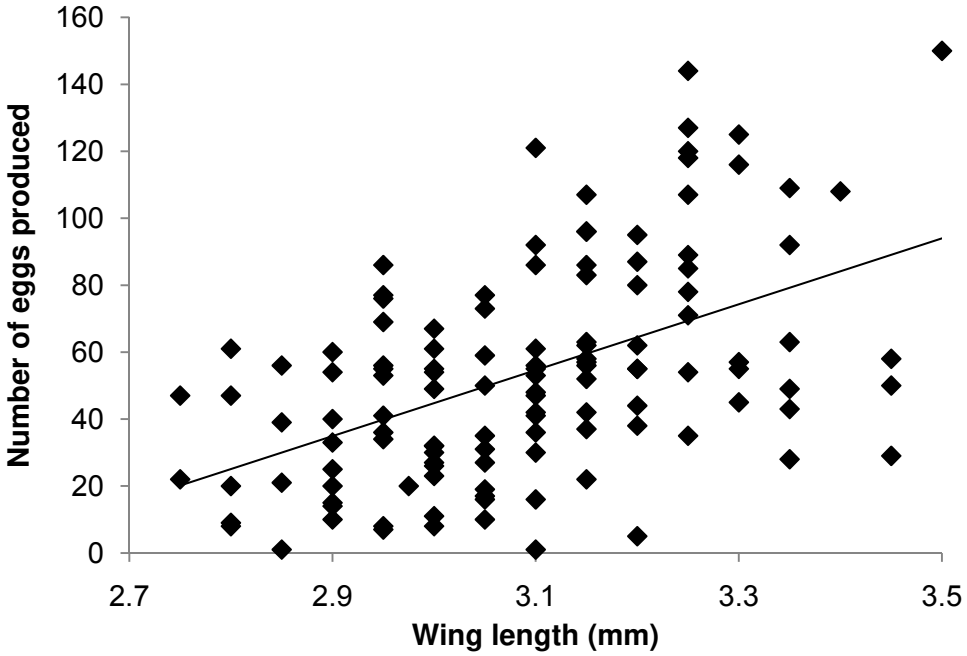

Figure 4.2. Number of eggs produced (laid plus retained) per mosquito in relation to wing length in *An. gambiae s.s.* mosquitoes. Line represents linear regression (adjusted r^2=0.25; p<0.0001)

4.5 Acknowledgements

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Part II

FISH

THE USE OF LARVIVOROUS FISH FOR MOSQUITO CONTROL IN WESTERN KENYA

Chapter 5

Abandoning small-scale fish farming in western Kenya leads to higher malaria vector abundance

Annabel F. V. Howard and Francois Omlin

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5.1 Abstract

Fishponds become abandoned due to lack of access to young fish and technical support, and faster economic returns from other activities. Certain conditions found in abandoned fishponds, such as absence of fish and presence of aquatic vegetation, are conducive to the proliferation of malaria vectors. We therefore conducted a district-wide fishpond census to determine the maintenance status of, and mosquito populations in, fishponds in Kisii Central District in western Kenya. 261 fishponds were found; 186 active (fish present) and 75 abandoned (fish absent). Vegetation was not significantly associated with the distribution of *Anopheles gambiae s.l., Anopheles funestus* or culicines (Diptera: Culicidae) in active or abandoned ponds. The presence of fish, however, correlated significantly with the distribution of all mosquito species, with significantly higher mosquito densities in abandoned fishponds. *Anopheles gambiae s.l.* was the most abundant mosquito species found in both active and abandoned ponds, being proportionally more abundant in the abandoned ponds when compared to other mosquito species. The relative proportion of *An. funestus* increased with altitude. Following the census the demand for fish to re-stock abandoned ponds rose by 67% when compared to the same time period in the previous year. This study highlights the potential public health problems associated with the abandonment of small-scale fish farming in the highlands of western Kenya.

5.2 Introduction

In sub-Saharan Africa, where most of the global malaria burden is borne, the main vectors are *Anopheles gambiae* Giles *s.l.* and *Anopheles funestus* Giles (Diptera: Culicidae). Although classically the larval habitats of *An. gambiae s.l.* are thought to be small, temporary, sunlit pools with algae (Gillies and Coetzee 1987, Gimnig et al. 2001), these species of mosquito have also been found in relatively large,

permanent, man-made water bodies in western Kenya (Lockhart et al. 1969, Fillinger et al. 2004, Mutuku et al. 2006b).

Small-scale fish farming has been continuously practiced in western Kenya for almost 50 years. Lockhart et al. (1969) found in 1959 that the 1,000 ponds they surveyed in the whole of western Kenya were well maintained, but by 1961 they found many fish ponds abandoned with over-grown edges and "only given the minimum amount of maintenance". This abandonment they attributed to "bad farming methods" which were producing smaller than anticipated yields, and because fish farming required more work than the farmers had expected. In addition, nowadays fishponds become abandoned for several other reasons, including the farmers switching their resources to invest in cash crops such as tea and coffee, demoralisation due to poaching, and lack of access to extension services from the Fisheries Department (FD) (for example help with harvesting and advice concerning pond construction and maintenance). Also, when the farmers harvest the ponds they remove all the fish including the small fry, in order to sell or eat them, which means that ponds become 'abandoned' for a while even if the farmers want to carry on fish farming.

Fish farming is still a favoured community activity in western Kenya today with new ponds being constructed and stocked (see Figure 2.2). It is promoted as a way to relieve the pressure on catches from Lake Victoria and as a source of income and protein for people living in rural areas (Government of Kenya 2002a, FAO Inland Water Resources and Aquaculture Service 2003). However, little attention is paid to possible dangers of abandoned fishponds in enhancing malaria transmission.

If a water body does not contain fish it is more likely to harbour mosquitoes than a water body stocked with fish (Petranka and Fakhoury 1991, Fletcher et al. 1992) with higher mosquito numbers in the fish-absent when compared to the fish-present areas (Wu et al. 1991, Prasad et al. 1993, Ritchie and Laidlaw-Bell 1994, Takagi et al. 1995). In addition, it has recently been found that *An. gambiae s.l.* pupal occurrence is positively correlated with both habitat size (Minakawa et al.

2005) and habitat stability (Mutuku et al. 2006b) in western Kenya. Most fishponds in western Kenya are large and contain water throughout the year and thus it would be expected that the pupal output from them would be very high.

Considering the above-mentioned issues, there is a possible danger that fishponds without fish and where the vegetation has been allowed to grow could harbour large numbers of malaria vectors. Therefore, we conducted a district-wide census to identify the number and maintenance status of fishponds in the Kisii Central District in western Kenya. The numbers of mosquito immatures found in these fishponds were counted to obtain a better understanding about the role of these numerous large permanent water bodies in the production of disease vectors during the dry season.

5.3 Materials and Methods

5.3.1 Study area

The census was carried out in the Kisii Central District of the Nyanza Province in western Kenya (Figure 5.1). This rural district is a highland area divided into seven administrative divisions covering a total area of 649 km^2 with altitudes of 1,400-2,200 m above sea level. Rainfall averages over 1,500 mm annually with two wet seasons (February to June and September to November). Mean annual maximum temperatures are 27ºC and 24ºC in the low and highland areas of the district respectively; the mean annual minimum temperatures are 16ºC and 14ºC in the low and highland areas respectively (Government of Kenya 2002a). The population density in the district is one of the highest in Kenya with a mean of 750 people per km^2 reaching a maximum of over 1,000 people per km^2 in some divisions. Malaria in this district is endemic but highly seasonal. *Anopheles gambiae s.l.* and *An. funestus* are the main malaria vectors in Kisii Central District.

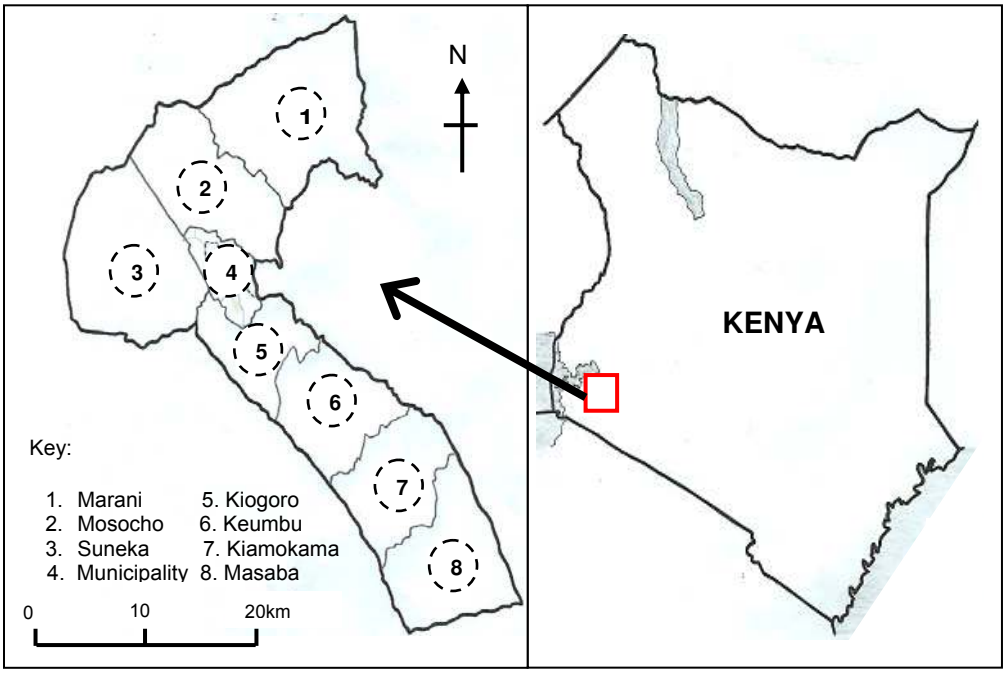

Figure 5.1. Map showing the location of each division in Kisii Central District, and the location of this district within Kenya

5.3.2 Fishpond census

A fishpond census was carried out in all divisions of Kisii Central District from the 5th January to the 6th February 2004 in collaboration with the Kenyan FD. The area including Kisii town (number 4 in Figure 5.1 and hereafter called Municipality) is part of Kiogoro, Mosocho and Suneka divisions but was treated separately.

The fishponds were classified as 'active' or 'abandoned' depending on the presence or absence of fish, respectively. The FD officer assessed whether the ponds contained fish by throwing a handful of fish food into the water and waiting to see whether fish rose to the surface, if no fish came to the surface after 10 minutes the pond was deemed to be without fish. The FD officer also visually identified the type of fish in active ponds as 'tilapiine', 'catfish' or 'other'. In some

cases the FD officer had recently had contact with the farmers and so already knew which fish type was in the pond. The ponds were further classified as well maintained (WM) if the banks were clear (A in Figure 5.2) and there was less than 10% vegetation coverage; not well maintained (NWM) indicated that the banks were not cleared of vegetation (B in Figure 5.2) and/or vegetation coverage of the pond was more than 10%.

Entomological assessments were carried out by taking five larval dips (2.5 litres total volume) randomly from the periphery of each pond, with at least one dip from each side. Anophelines and culicines were distinguished and the anopheline mosquitoes were identified to species level using a morphological key (Gillies and Coetzee 1987). The surface area of the ponds was measured and the owners of the ponds were educated by the FD officer about fishpond maintenance and the associated risk of mosquitoes breeding in abandoned fishponds.

5.3.3 Statistical analysis

After testing for equal/unequal variance, two-sample Student's t-tests were carried out to investigate the differences between WM and NWM fishponds within the active and abandoned pond classes for each mosquito type. Two-sample t-tests were also used to analyse any differences within each mosquito type between the active and abandoned pond types. Chi-square tests were used to investigate the differences between the active and abandoned ponds with respect to the number of ponds containing mosquitoes of any type, *An. gambiae s.l., An. funestus*, culicines, and the number of WM ponds. Within each pond type differences between the mosquito types were analysed using one-way ANOVA analysis. Statistical analysis was carried out at the 5% significance level using the SAS 9.1 software (SAS Institute Inc. 2004).

Figure 5.2. Photographs showing the clear banks of a well maintained fishpond (A) and the overgrown banks of a not well maintained fishpond (B)

5.4 Results

5.4.1 Differences between WM and NWM ponds

Of the 186 active ponds found, 148 (80%) were WM, whereas just 13 (17%) of the 75 abandoned ponds were WM (Table 5.1); this difference was highly significant (χ^2=87.60, df=1, p<0.0001). The observed mean surface area of the WM and NWM ponds was not significantly different (t=1.10, df=184, p=0.27 for active; t=0.8, df=73, p=0.42 for abandoned ponds). Within the active ponds the proportion of *An. gambiae s.l.* in the NWM ponds decreases when compared to the WM ponds, with corresponding increases in the proportions of *An. funestus* and culicines. Within both the active and abandoned fishpond classes there were no significant differences for any of the mosquito types when comparing the WM and NWM ponds (Table 5.2). As such the active WM and NWM and the abandoned WM and NWM data were pooled for further analysis.

Table 5.1. Number (N) and surface area (mean ± SE) of the different pond types and the relative mosquito abundance (%)

Pond	Veg.	N	Area (m^2)	*An. gam.%*	*An.fun.%*	Culicines%
Active	WM	148	130.0 ± 7.58	54.7	28.7	16.6
	NWM	38	148.9 ± 16.78	32.7	42.5	24.8
	Total	186	133.9 ± 6.94	48.1	32.9	19.0
Aband.	WM	13	118.2 ± 22.64	65.2	21.7	13.1
	NWM	62	143.2 ± 11.03	60.7	23.8	15.5
	Total	75	138.9 ± 9.93	61.7	23.4	14.9

Veg. = Vegetation; *An. gam.* = *An. gambiae s.l.*; *An. fun* = *An. funestus*; Aband. = Abandoned; WM = Well Maintained; NWM = Not Well Maintained

Table 5.2. Mean ± SE relative immature mosquito population densities in well maintained (WM) and not well maintained (NWM) fishponds

Pond	Mosquito type	WM	NWM	t-stat	df	p
Active	*An. gambiae s.l.*	1.68 ± 0.21	1.66 ± 0.47	0.04	184	=0.97
	An. funestus	0.88 ± 0.13	2.16 ± 0.64	1.96	40	=0.06
	Culicines	0.51 ± 0.10	1.26 ± 0.59	1.28	39	=0.21
Aband.	*An. gambiae s.l.*	15.69 ± 3.03	11.18 ± 3.74	0.94	53	=0.35
	An. funestus	5.23 ± 3.77	4.39 ± 0.78	0.22	13	=0.83
	Culicines	3.15 ± 1.14	2.85 ± 0.92	0.20	30	=0.84

5.4.2 Differences between active and abandoned ponds

There was no significant difference between active and abandoned ponds with respect to the number of ponds containing mosquitoes of any type (χ^2=2.62, df=1, p=0.11). However, when looking at individual mosquitoes species, significantly more abandoned ponds contained *An. gambiae s.l.* (χ^2=13.83, df=1, p<0.0002), *An. funestus* (χ^2=5.68, df=1, p<0.02) and culicines (χ^2=6.65, df=1, p<0.01). In addition, the proportion of *An. gambiae s.l.* was higher in the abandoned ponds (Table 5.1). The mean number of *An. gambiae s.l.* found in the abandoned fishponds was also an order of magnitude higher than the mean number found in active fishponds and there were larger numbers of *An. funestus* and culicines in abandoned when compared to active fishponds. These differences were all statistically highly significant (Table 5.3), however, there was no significant difference between the mean surface areas of the active and abandoned fishponds (t=0.43, df=259, p=0.67).

5.4.3 Differences between mosquito types within the pond types

The ANOVA results showed a significant effect of the mosquito species on the number of mosquito immatures in both the active (F=8.92, df=2,555, p<0.0002) and abandoned (F=6.20, df=2,222, p<0.003) ponds. Therefore, Student-Newman-Keuls (SNK) multiple comparison analysis was carried out to investigate which species were significantly different within each pond type (Table 5.3).

5.4.4 Fish type

In the 151 active fishponds (122 WM, 29 NWM) where the fish types were identified, 147 (97%) contained tilapiine fish (121 WM, 26 NWM). Of the WM ponds with tilapiine fish, 27% had no mosquitoes in five dips compared to 12% of the NWM ponds. This was not significant using the Fisher's exact test (p=0.13).

Table 5.3. Mean ±SE immature mosquito population densities in active and abandoned fishponds after pooling the WM and NWM data

Mosquito type	Active	Abandoned	t-stat	df	p
An. gambiae s.l.	1.67 ± 0.19 **A**	11.96 ± 3.1 **A**	3.27	75	<0.002
An. funestus	1.14 ± 0.17 **B**	4.54 ± 0.90 **B**	3.70	79	<0.0004
Culicines	0.66 ± 0.14 **C**	2.90 ± 0.78 **B**	2.83	79	<0.006

The same letters in a column indicate no significant difference as calculated by SNK multiple comparison analysis; there is no relationship between the letters within the rows.

5.4.5 Levels of fishpond abandonment in the different divisions

A mean of 32 fishponds per division were found (23 active; 9 abandoned) (Table 5.4). In all divisions there were more active than abandoned fishponds. Suneka division had the highest rate of abandonment. There was no correlation between the percent active and abandoned ponds that were NWM within the divisions. Because the pond numbers in the different divisions varied widely and in some divisions were very low, no statistical tests were carried out between the different divisions.

5.4.6 Mosquito types per division

The proportion breakdown of the different mosquito types per division can be seen in Figure 5.3. In the abandoned ponds *An. gambiae s.l.* predominated especially in Municipality, Mosocho, Suneka and Marani, while in both the active and abandoned ponds the relative proportion of *An. funestus* was high in Keumbu, Masaba and Kiamokama and to a lesser extent in Kiogoro.

Table 5.4. Divisional breakdown of the number of fishponds found and the proportion of active and abandoned fishponds not well maintained

Division		Pond type (N)				NWM	
Name	Area (km$^{2)}$	Active	Aband.		Total	Active	Aband.
Municipality	29	18	8	(30.8%)	26	38.9%	75.0%
Mosocho	105	10	7	(41.2%)	17	20.0%	57.1%
Suneka	126	33	24	(42.1%)	57	18.2%	79.2%
Marani	124	37	5	(11.9%)	42	10.8%	100%
Kiogoro	61	46	11	(19.3%)	57	17.4%	90.9%
Keumbu	71	13	8	(38.1%)	21	23.1%	100%
Masaba	90	11	3	(21.4%)	14	45.5%	66.7%
Kiamokama	72	18	9	(33.3%)	27	16.7%	88.9%

NWM = Not well maintained; Aband. = Abandoned

5.4.7 Community response to the census

Following the census the FD reported that the demand for both fish and help with renovating and restocking abandoned fishponds rose. For the first six months of 2004 there was a 67% increase in the demand for fish when compared to the same time period of 2003.

5.5 Discussion

As in previous studies (Petranka and Fakhoury 1991, Fletcher et al. 1992) we found that more fish-absent (abandoned) than fish-present (active) fishponds

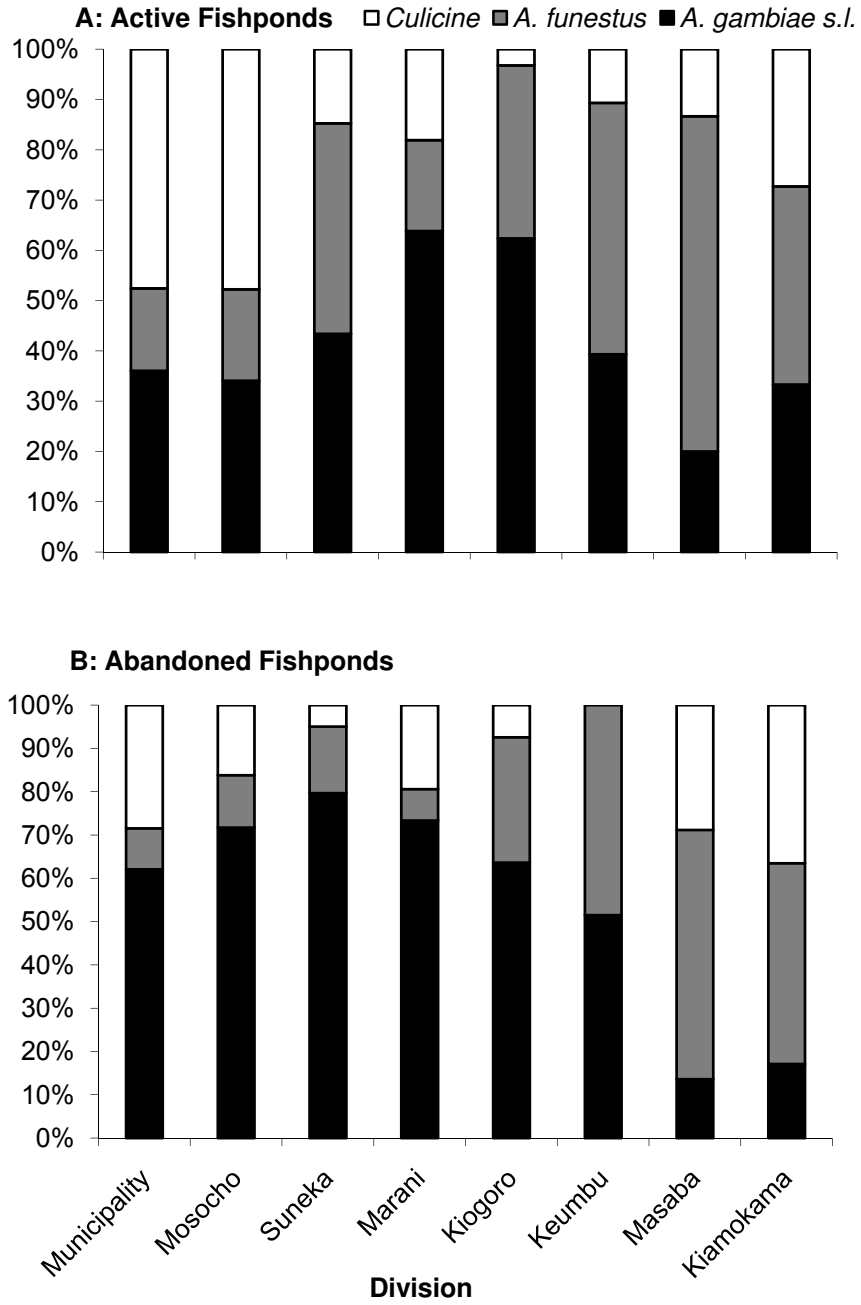

Figure 5.3. Proportional breakdown of mosquito types per division in active (A) and abandoned (B) fishponds

contained mosquitoes, although this difference was not significant when all the different mosquito types were combined. However, when the mosquito types were considered separately, significantly more abandoned ponds contained mosquitoes of each type. This may be because *An. gambiae s.s.* avoids ovipositing in water that contains competitors (Munga et al. 2006). In this respect *An. funestus* may be seen as a competitor by *An. gambiae s.l.*, and it is also possible that a similar mechanism exists in *An. funestus*.

Our results indicate that fish significantly influence the distribution of anophelines and culicines in the study area. This phenomenon has also been found in previous studies in other geographical locations (Wu et al. 1991, Prasad et al. 1993, Ritchie and Laidlaw-Bell 1994). Fish can influence mosquito numbers both directly and indirectly. Larvivorous fish directly control mosquito numbers through predation (Asimeng and Mutinga 1993). In over 80% of the active fishponds the fish type was successfully identified, and in these cases, 97% of the fish belonged to the tilapiine sub-family. Although not identified in this study, the main tilapiine fish species farmed in the study area are *Oreochromis niloticus* (Perciformes: Cichlidae) and *Tilapia zillii* (Perciformes: Cichlidae). Under laboratory conditions both of these species have been shown to eat mosquito larvae, with one individual fish eating on average 300 mosquito larvae in just 15 minutes (Asimeng and Mutinga 1993), while in the field *O. niloticus* has been shown to eat both mosquito eggs and larvae (el Safi et al. 1985). We have also demonstrated the ability of *O. niloticus* to control mosquito numbers in WM fishponds under field conditions in Kisii Central District; we found that the fish caused >94% reduction in malaria vectors in only 15 weeks (Howard et al. 2007) (**Chapter 6**). Fish indirectly influence mosquito numbers by causing ovipositing mosquitoes to selectively avoid fish-containing water bodies (Petranka and Fakhoury 1991, Ritchie and Laidlaw-Bell 1994, Angelon and Petranka 2002). Although to our knowledge this has not been proven to occur in our study area.

Previous work has shown no relationship between vegetation and either *An.*

gambiae s.l. (Minakawa et al. 1999) or culicines (Minakawa et al. 1999, Fillinger et al. 2004), and this was also found in this study. Despite the previously published positive association of *An. funestus* and aquatic vegetation (Lockhart et al. 1969, Gimnig et al. 2001) we found that vegetation did not significantly influence the distribution of *An. funestus* in this study.

Ndenga et al. (2006), in a study carried out at the same time as ours, concluded that *An. gambiae s.s.* predominated in the highland areas while *An. funestus* predominated in a lowland area of western Kenya. They did, however, observe that in the lowland area there were more *An. funestus* favourable breeding habitats. Their highland area was Marani division (Kisii Central District) and they found 79.1% of the malaria vectors were *An. gambiae s.s.* (with all *An. gambiae s.l.* identified as *An. gambiae s.s.*). In Marani division we found *An. gambiae s.l.* constituted 63.9% and 73.4% of the active and abandoned fishpond mosquitoes, respectively, and in both types of fishpond it was certainly the predominant mosquito species. Marani, however, is a relatively low-lying part of Kisii Central District (Government of Kenya 2002a). In Kiogoro, Keumbu, Masaba and Kiamokama, which are located at a higher altitude than the other divisions, we found the proportion of *An. funestus* to be relatively high, which is likely to be an effect of altitude as this observation was made in both the active and abandoned fishponds. A study in Tanzania showed that *An. funestus* predominated in highland when compared to lowland areas throughout the year, with the reverse true for *An. gambiae s.l.* (Maxwell et al. 2003). This possible association of *An. funestus* with altitude is interesting and should be investigated further.

There was much variation in the rates of abandonment of fishponds between the divisions. Kiamokama is rural and does not have good access to the market or FD extension services which may be why the rate of abandonment is higher than average. In contrast Kiogoro has easy access to the market and extension services and its rate of abandonment is below average. Municipality, which has good access to the market and FD extension services, also has a high level of

abandonment and this may be because in the town there are many other income generating opportunities which have a quicker return than fish farming. Suneka, Mosocho and Keumbu have the highest rates of abandonment. In all of these three divisions some incentives were offered to start fish farming and this support has subsequently been withdrawn.

Kisii Central District was reported to have 554 fishponds in the year 2000 (Government of Kenya 2002a). The number of fishponds we found in this study was lower than anticipated possibly due to difficulties in accessibility. Kisii Central District is a mainly rural district with many areas inaccessible by vehicle. The topography is hilly and there are lots of trees, so fishponds could not easily be visually identified. Since the census finished, we have had much information regarding newly constructed and stocked fishponds, and about existing ponds that we missed during the census, indicating that at present fishpond numbers in the district are much higher than reported here.

There were certain other limitations of this study, namely that it represents the situation at just one time point during the dry season. A comparative study during the wet season would be interesting to see if the dynamics of the different mosquito species changed at all, especially as we have found that mosquito numbers in fishponds decrease in the wet season (Howard et al. 2007) (**Chapter 6**). Also, information about the larval instar and pupal numbers would have been interesting to see if there was any difference between the active and abandoned ponds with respect to the instars present. Similarly, information about productivity of the fishponds in the form of adult mosquito emergence could have provided much information on the actual public health risk involved. Many of these issues were not undertaken due to the time constraints involved in trying to conduct a single time point census over such a large area where movement via roads is slow.

One of the main points from the 2005 Abuja Declaration on Sustainable Fisheries and Aquaculture in Africa is to develop aquaculture, and aquaculture worldwide has increased exponentially (FAO Inland Water Resources and Aquaculture

Service 2003, Keiser and Utzinger 2005). Our findings indicate that aquaculture development should be accompanied by adequate public health education so that fish farmers know the potential risks of abandoning their ponds. The main problem at present is the lack of information and knowledge that the farmers have about these risks. We are working with the FD towards reactivating the abandoned fishponds in this area and the FD are educating the fish farmers they come into contact with about the dangers of abandoned ponds.

Our data indicate that fishponds could be of considerable public health relevance if abandoned, particularly because the human population in Kisii Central District is around 750 people per km^2. As these fishponds contain water throughout the year this is potentially a year-round problem. This study suggests that for effective mosquito control, and associated malaria prevention, all fishponds should be stocked with larvivorous fish. With new fishponds continually being constructed in the area we think that this is an important issue to address. Therefore we recommend the following:

- Public health officers and the FD should create wider awareness about the problem of abandoned fishponds among the fish farmers.
- Draining the water from abandoned ponds is not enough for mosquito control because in this area of Kenya there is abundant rainfall that will gather in any depression in the ground. Therefore abandoned ponds should be filled in.
- Fish should not be over-fed by their owners, because then they do not eat the mosquitoes and vegetation in the pond.

5.6 Acknowledgements

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Chapter 6

Malaria mosquito control using edible fish in western Kenya: preliminary findings of a controlled study

Annabel F. V. Howard, Guofa Zhou, Francois Omlin

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6.1 Abstract

Biological control methods are once again being given much research focus for malaria vector control. This is largely due to the emerging threat of strong forms of resistance to pesticides. Larvivorous fish have been used for over 100 years in mosquito control and many species have proved effective. In the western Kenyan highlands, the larvivorous fish *Oreochromis niloticus* L. (Perciformes: Cichlidae) (formerly *Tilapia nilotica*) is commonly farmed and eaten but has not been tested previously in the field for malaria mosquito control. This fish was introduced into abandoned fishponds at an altitude of 1,880 m and the effect measured over six months on the numbers of mosquito immatures. For comparison an untreated control pond was used. During this time, all ponds were regularly cleared of emergent vegetation and fish re-stocking was not needed. Significant autocorrelation was removed from the time series data, and t-tests were used to investigate within a pond and within a mosquito type any differences before and after the introduction of *O. niloticus*. Mulla's formula was also used on the raw data to calculate the percentage reduction of the mosquito larvae. After *O. niloticus* introduction, mosquito densities immediately dropped in the treated ponds but increased in the control pond. This increase was apparently due to climatic factors. Mulla's formula was applied which corrects for that natural tendency to increase. The results showed that after 15 weeks the fish caused a more than 94% reduction in both *Anopheles gambiae s.l.* and *Anopheles funestus* (Diptera: Culicidae) in the treated ponds, and more than 75% reduction in culicine mosquitoes. There was a highly significant reduction in *An. gambiae s.l.* numbers when compared to pre-treatment levels. This study reports the first field trial data using *O. niloticus* for malaria mosquito control and shows that this species, already a popular food fish in western Kenya, is an apparently sustainable mosquito control tool which also offers a source of protein and income to people in rural areas. There should be no problem with acceptance of this malaria control method since the local communities already farm this fish species.

6.2 Introduction

Mosquito control relies heavily on synthetic pyrethroids. Concern about the threat of strong forms of resistance (Hargreaves et al. 2000) has stimulated renewed interest in alternative control methods including biological control and biopesticides. At present these methods are only operational against mosquito immatures (Fletcher et al. 1992, Takagi et al. 1995, Kay et al. 2002, Mittal 2003), the best known being the use of *Bacillus thuringiensis* var. *israelensis* (Bti). Bti is effective against mosquito larvae (Mittal 2003) but cannot control the pupal stage, frequent repeat applications are needed (Gunasekaran et al. 2004) and it is expensive. Another biocontrol method, the use of larvivorous fish in appropriate water bodies, has been used in mosquito control for over 100 years (Bay 1967) and can also be effective (Fletcher et al. 1992, Asimeng and Mutinga 1993, Prasad et al. 1993, Mohamed 2003). However, larvivorous fish offer advantages when compared to Bti. Fish feed on mosquito pupae and are generally self-sustaining, so in most cases do not require repeat applications. One disadvantage is that larvivorous fish can only be used under certain conditions conducive to their survival.

Almost 200 fish species are known to feed on mosquito larvae (Jenkins 1964). *Oreochromis niloticus* L. (Perciformes: Cichlidae) (formerly *Tilapia nilotica*) is a native African fish possessing mosquito control properties known since 1917 (Austen 1919). To our knowledge though, no field data has been published on its use for mosquito control. Under laboratory conditions this fish species has been shown to be larvivorous (Kusumawathie et al. 2006) with a 'marked interest in mosquito larvae' (Asimeng and Mutinga 1993). The fry actively pursue mosquito immatures (Trewavas 1983), however, when the fish are greater than 150 mm in length they prefer eating macrophytes (el Safi et al. 1985). Therefore, larger fish eat the plant material in which the mosquito immatures hide, allowing the fry to find them. This fish species, commonly farmed by people in western Kenya as a source of protein and income, is a prolific breeder spawning every few weeks.

This study reports the first field trial using *O. niloticus* for mosquito control. Previously we found that abandoned (fish absent) fishponds had alarmingly high mosquito larval densities when compared to fishponds still containing fish (Howard and Omlin 2008) (**Chapter 5**). We therefore investigated the long-term impact on mosquito densities of introducing *O. niloticus* into abandoned fishponds.

6.3 Materials and Methods

6.3.1 Study area

The study area was in Kisii Central District of western Kenya. The intervention site is 00°42 S, 34°46 E, at an elevation of 1,880 m above sea level with a population density of >1,000 people per km^2 (Government of Kenya 2002a). Malaria in the area is endemic but highly seasonal with >2,000 paediatric cases annually in the district hospital (Hay et al. 2002). The primary malaria vectors in the area are *Anopheles gambiae* Giles *s.l.* and *Anopheles funestus* Giles. Rainfall averages over 1,500 mm annually with two wet seasons (February to June and September to November) and the mean annual maximum and minimum temperatures are 24°C and 14°C respectively (Government of Kenya 2002a). Climate data for the study period was obtained from the Kenya Agricultural Research Institute and is shown in Figure 6.1.

6.3.2 Field intervention

The site has three abandoned fishponds within 150 m of each other. Pond A (104 m^2 surface area) served as the untreated control and ponds C (128 m^2) and D (72 m^2) were assigned for stocking with *O. niloticus*; each pond had a depth of 30 cm. These fishponds had been re-constructed under the instruction of an officer from the Kenyan Fisheries Department (FD). Entomological assessments were carried

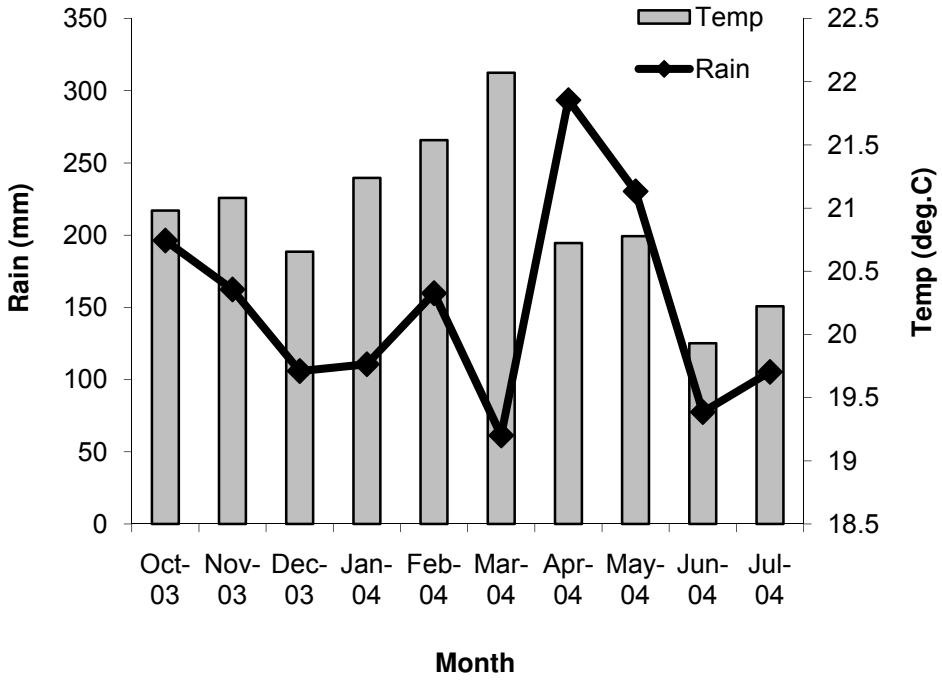

Figure 6.1. Mean monthly temperature and total monthly rainfall for the study area

out by taking five larval dips (2.5 litres total volume) randomly from the edges of each pond, with at least one dip from each side. These assessments were carried out 5-7 days a week and began on the 1st October 2003. Anophelines and culicines were distinguished, with anophelines identified to species level using a morphological key (Gillies and Coetzee 1987). On the 14th January 2004, one-to-two month old *O. niloticus* from the local FD hatchery in Kisii town were stocked in ponds C and D at a rate of two fish per m^2 pond surface area. FD representatives instructed the fishpond owners on fish husbandry and pond maintenance. The three ponds were cleared of vegetation on a weekly basis and treated identically during the nine month study period. The fish were neither harvested nor the ponds restocked.

6.3.3 Statistical analysis

Analysis was carried out on the data 15 weeks before and 15 weeks after fish introduction into ponds C and D. We used the one-lag autoregression model to determine the autocorrelation of the time series data. Significant autocorrelation was removed along with the deterministic drift term. We then used paired t-tests to see if the two treated ponds were significantly different before fish introduction. If there was no difference the data from the ponds were pooled. We also used t-tests to investigate within the pond and within the mosquito type any differences before and after fish introduction. All tests were carried out at the 5% significance level.

Using the raw data, the percentage reduction of mosquito immatures in ponds C and D after fish introduction was calculated using Mulla's formula:

$$\% \text{ reduction} = 100 - ((C_1/T_1)(T_2/C_2))100$$

where C_1 is the average number of larvae pre-treatment in the control pond, T_1 is the average number of larvae pre-treatment in the treated ponds, T_2 is the average number of larvae post-treatment in the treated ponds, and C_2 is the average number of larvae post-treatment in the control pond (Mulla et al. 1971). This formula corrects for any changes seen in the control pond that would presumably also have occurred in the treated ponds in the absence of the intervention.

6.4 Results

The *An. gambiae s.l.* numbers in ponds A, C and D for 15 weeks prior to and 41 weeks after *O. niloticus* introduction into ponds C and D are presented in Figure 6.2. Ten days after fish introduction, no mosquitoes were found in pond C and a clear difference can be seen between ponds A and C for the next six months. Pond D shows a similar pattern.

The mean immature mosquito densities before autocorrelation removal are

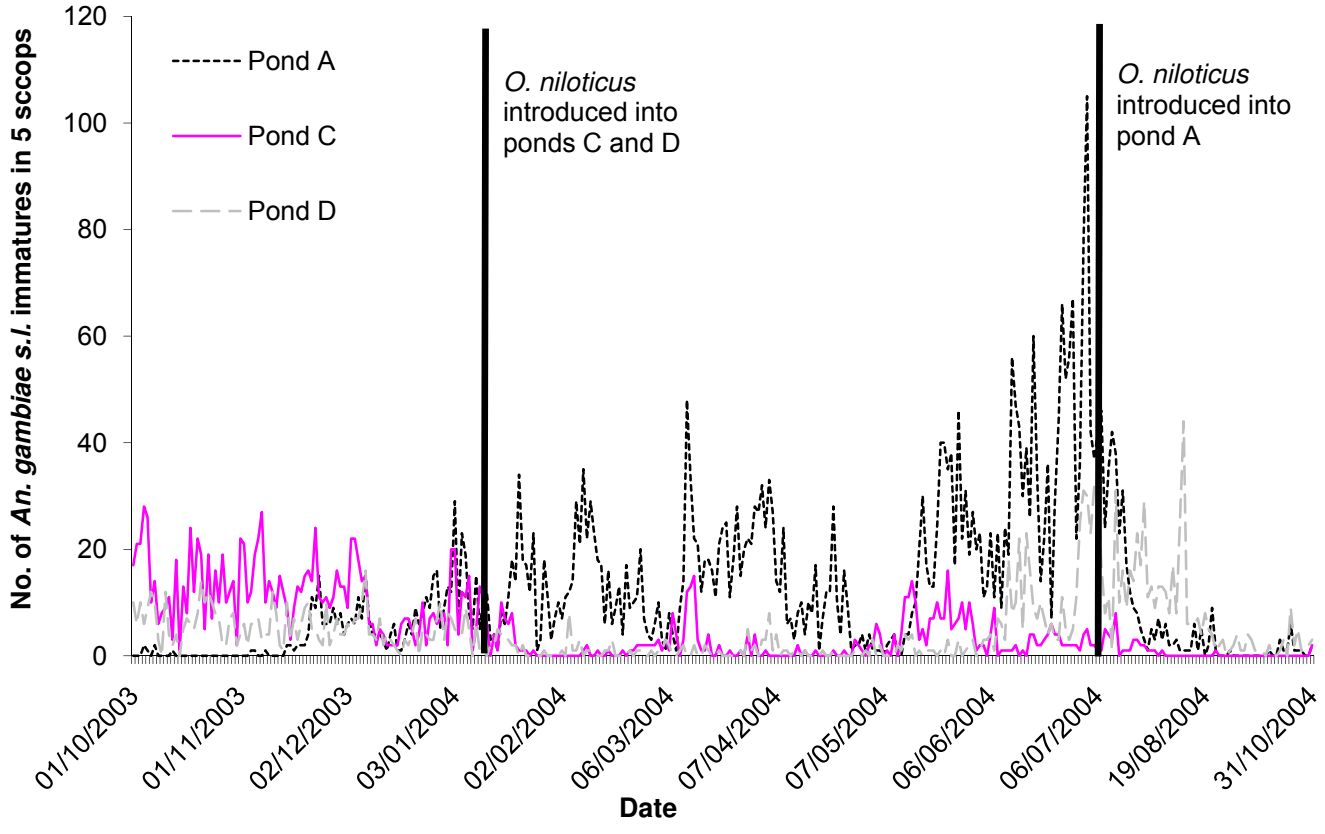

Figure 6.2. *An. gambiae s.l.* numbers in the control (pond A) and treated (ponds C and D) ponds before and after fish introduction

presented in Table 6.1, along with the % reduction as calculated using Mulla's formula (Mulla et al. 1971). It can be seen that after fish introduction, the numbers of all mosquito types increased in the control pond, and decreased in the treated ponds. High percentage reductions were found for *An. gambiae s.l*. and *An. funestus*. For culicines it was lower but the reduction was still >75%.

Significant autocorrelation was detected in all ponds for all mosquito species except for culicines in pond C. However, the first two data points for culicines in pond C were removed from the analysis in order to use the same number of data points as ponds A and D. No significant differences between ponds C and D for *An. gambiae s.l.* or *An. funestus* before fish introduction were found so the data were pooled. After autocorrelation removal, when comparing within a pond the pre- and post-intervention data, fish introduction caused highly significant reductions of *An. gambiae s.l.* in the treated ponds ($t=3.81$, $df=127$, $p<0.0002$) and culicines in pond C ($t=4.16$, $df=128$, $p<0.0001$), and a significant reduction of culicines in pond D ($t=1.97$, $df=162$, $p<0.05$).

Table 6.1. Mean ±SE immature mosquito densities before and after *O. niloticus* introduction into ponds C and D

	Pond A		Pond C			Pond D		
Mosquito	**Before**	**After**	**Before**	**After**	**% Red.**	**Before**	**After**	**% Red.**
An. gambiae	4.5±0.6	14.1±0.9	11.4±0.7	1.5±0.3	95.8	5.4±0.4	1.0±0.2	94.1
An. funestus	0.1±0.1	2.5±0.5	0.6±0.1	0.4±0.1	98.3	0.6±0.1	0.6±0.1	97.5
Culicines	2.4±0.4	4.5±0.4	2.8±0.5	0.7±0.2	86.7	1.3±0.2	0.6±0.1	75.4

Pond A was the control pond, ponds C and D were stocked with fish; 'Before' = the 15 weeks before fish introduction into ponds C and D; 'After' = the 15 weeks after fish introduction into ponds C and D; % Red. = Percentage reduction was corrected for natural increases in the control pond using Mulla's formula (Mulla et al. 1971).

An. funestus numbers in the treated ponds decreased but not significantly (t=1.13, df=129, p=0.26). In the control pond the mosquito numbers increased for all species.

In view of the high *An. gambiae s.l.* densities in pond A, we introduced *O. niloticus* into this pond once the experiment was complete. These densities dropped from 105 mosquitoes in five dips just before fish introduction, to one mosquito in five dips two weeks later and remained low for the next three months (Figure 6.2). However, without a contemporary untreated control we cannot be sure this was solely because of the fish.

6.5 Discussion

Our field data demonstrates for the first time, that the introduction of *O. niloticus* into fishponds immediately and significantly reduces the numbers of *An. gambiae s.l.* and culicine larvae in treated ponds. Fifteen weeks after fish introduction, the impact on both anopheline species was a >94% reduction after correction for the natural increase expected. By contrast, Mohamed (2003) reported that *Oreochromis spilurus spilurus* introduced into water storage containers in Somalia showed a mean reduction of 52.8%, while *Gambusia affinis* produced a 87.8% decline in mosquito larvae in rice fields (Prasad et al. 1993). However, these results represent both different fish species and ecological settings.

The large percentage reductions in the treated ponds, as calculated with the raw data using Mulla's formula (Mulla et al. 1971), was a combined effect of the decrease of mosquito numbers in the treated ponds and the increase in the control pond. Fifteen weeks after fish introduction into ponds C and D there was an increase of all mosquito species in the control pond. This was most likely due to low rainfall leading to a reduction in the number of alternative oviposition sites. When the rainfall increased in April, the number of mosquitoes in the control pond

decreased. This negative correlation of mosquito larval densities with rainfall has been previously found in Kenyan rice fields (Asimeng and Mutinga 1993).

The decrease of the mosquito numbers in the treated ponds might be directly (by predation) and/or indirectly (by oviposition avoidance by mosquitoes) due to the fish. Evidence that the fish were directly responsible comes from observed minor peaks in the mosquito densities in ponds C (from 13th May) and D (from 8th June) that corresponded with the time when the fish were mature enough to start reproduction. When reproducing, neither male nor female *O. niloticus* feed (Trewavas 1983), which would explain the temporary peaks, contrary to the overall downward trend. A tendency of ovipositing mosquitoes to avoid ponds containing fish has previously been found with *Anopheles punctipennis* (Petranka and Fakhoury 1991), and culicine mosquitoes (Ritchie and Laidlaw-Bell 1994, Angelon and Petranka 2002). However, in a separate study of 261 fishponds we found no significant difference between the number of fish-present and fish-absent fishponds containing mosquito immatures (Howard and Omlin 2008) (**Chapter 5**). This suggests that mosquitoes do not avoid fish-containing water in this area.

Given the already proven larvivorous behaviour of *O. niloticus*, the peaks of mosquitoes during fish reproduction, the findings in the separate study, and also taking into account the climatic relationship of the mosquito increase in the control pond, it seems likely that the fish are directly controlling the mosquito numbers in the treated ponds through predation.

Anopheles funestus was not significantly decreased after fish introduction. The large percentage decrease calculated is a result of the 40-fold increase in the control pond, indicating a strong tendency for natural increase in the local population. The fish apparently ate enough *An. funestus* larvae to counterbalance this natural increase, but not enough to produce an overall reduction.

The fact that we still recorded larvae in the treated ponds does not mean these ponds were still producing adult mosquitoes. From other sites in Kisii Central

District, we have noticed disproportionate numbers of first and second instar mosquito larvae in fishponds containing fish, indicating that fish are more likely to eat the older, larger instars. This was also found with other fish species in Somalia where after fish introduction only first and second instar larvae were present (Mohamed 2003), and in laboratory and field studies in China (Wu et al. 1991).

In the year 2000, Kisii Central District was reported to have 554 fishponds in an area of 649 km^2 (Government of Kenya 2002a) while the neighbouring district of Nyamira was reported to have 1,046 fishponds in 896 km^2 (Government of Kenya 2002b). It is likely that these are under-representations of the actual fishpond numbers in the two rural districts as the topography is hilly with poor road networks, which make locating fishponds difficult. Given the large size of the fishponds and the fact that they contain water all year round, they could be considered a fairly significant producer of malaria vectors in this area of western Kenya. Unfortunately our results do not show the proportion of the adult mosquito population that is produced by the fishponds, relative to the more classic *An. gambiae s.l.* immature habitats such as small transient pools of water (Gimnig et al. 2001) which are unsuitable for *O. niloticus*. As such we are unable to say how effective this control method would be in reducing the adult mosquito population in a given area. However, our results show that *O. niloticus* fish were so effective in reducing immature mosquito populations in the fishponds studied, that there is likely to be a noticeable effect on the adult mosquito population in the area.

Benefits of larvivorous fish are that the mosquito larvae cannot build up a physiological resistance. Also, fish populations are generally self-sustaining and do not depend on the presence of larvae. By contrast survival of other biological control agents is often dependent on the mosquito population not being entirely eliminated (Wright et al. 1972). In addition some *Anopheles* larvae have significantly prolonged developmental times in the presence of fish and emerge as smaller adults (Bond et al. 2005). Smaller females, in turn, have significantly reduced host seeking (Takken et al. 1998) making them less efficient malaria

vectors. As well as protection from mosquito-borne diseases such as malaria, *O. niloticus* has additional benefits. The fish are relatively inexpensive and six months after stocking, the larger fish can be harvested providing a sustainable source of income and protein to rural farmers. This fish is already farmed and eaten in this region of Kenya so acceptance by both the local communities and the administrative sectors should pose no problem.

Larval control has long been neglected. However, it can be an effective control tool due to the low mobility of larval mosquitoes (Killeen et al. 2002), especially where the principle breeding habitats are man-made (Carlson et al. 2004, Fillinger et al. 2004, Mutuku et al. 2006b) and can be easily identified (Walker and Lynch 2007). To verify the findings in this study, in villages both with and without fish introductions, it is necessary to monitor adult mosquito densities and malaria incidence to confirm the use of *O. niloticus* as a malaria control tool.

In conclusion, our results indicate that *O. niloticus* can dramatically reduce mosquito larval densities in fishponds for at least six months and that this reduction is directly through predation. The relative population density of *An. gambiae s.l.*, a very efficient malaria vector, was reduced by 94% and this reduction was statistically highly significant.

6.6 Acknowledgements

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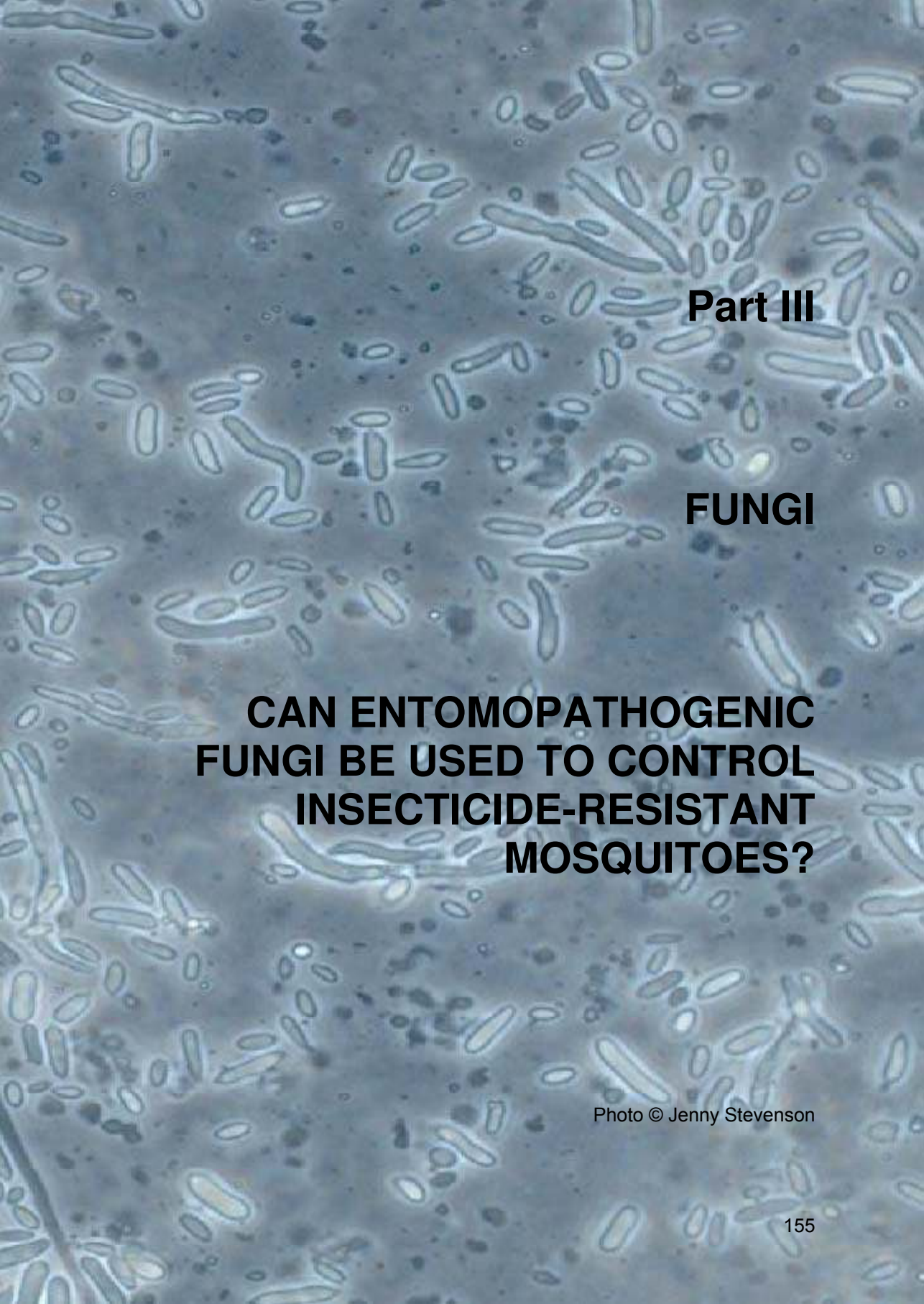

Part III

FUNGI

CAN ENTOMOPATHOGENIC FUNGI BE USED TO CONTROL INSECTICIDE-RESISTANT MOSQUITOES?

Photo © Jenny Stevenson

Chapter 7

Pyrethroid resistance in *Anopheles gambiae s.s.* leads to increased susceptibility to the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*

Annabel F.V. Howard, Constantianus Koenraadt,

Marit Farenhorst, Bart Knols, Willem Takken

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7.1 Abstract

Entomopathogenic fungi are being investigated as a new mosquito control tool because insecticide resistance is preventing successful mosquito control in many countries, and new methods are required that can target insecticide-resistant malaria vectors. Although laboratory studies have previously examined the effects of entomopathogenic fungi against adult mosquitoes, most application methods used cannot be readily deployed in the field. Because the fungi are themselves biological organisms that can be affected by the formulation they are suspended in and substrate onto which they are placed, it is important to test potential application methods that will not adversely affect them and which can also be used in the field. The two objectives of this study were to investigate any differences in fungal susceptibility between an insecticide-resistant and insecticide-susceptible strain of *Anopheles gambiae* Giles *sensu stricto,* and to test a potential field application method with respect to the viability and virulence of two fungal species. Pieces of white polyester netting were dipped in *Metarhizium anisopliae* ICIPE-30 or *Beauveria bassiana* IMI391510 mineral oil suspensions. These were kept at 27±1°C, 65±10% RH and the viability of the fungal conidia was recorded at different time points. Tube bioassays were used to infect insecticide-resistant (VKPER) and insecticide-susceptible (SKK) strains of *An. gambiae s.s.*, and survival analysis was used to determine effects of mosquito strain, fungus species or time since fungal treatment of the net. The insecticide-resistant VKPER strain was significantly more susceptible to fungal infection than the insecticide-susceptible SKK strain. Furthermore, *B. bassiana* was significantly more virulent than *M. anisopliae* for both mosquito strains, although this may be linked to the different viabilities of these fungal species. The viability of both fungal species decreased significantly one day after application onto polyester netting when compared to the viability of conidia remaining in suspension. That the insecticide-resistant mosquito strain was susceptible to both species of fungus indicates that entomopathogenic fungi can be used in resistance management and integrated vector management programmes to target insecticide-resistant mosquitoes.

Although fungal viability significantly decreased when applied to the netting, the effectiveness of the fungal treatment at killing mosquitoes did not significantly deteriorate. Field trials over a longer trial period need to be carried out to verify whether polyester netting is a good candidate for operational use, and to see if wild insecticide-resistant mosquitoes are as susceptible to fungal infection as the VKPER strain.

7.2 Introduction

It is estimated that in 2008 there were 243 million cases of malaria and 863,000 deaths (World Health Organisation 2009). Clearly, mosquito-borne diseases are still a major health risk, particularly in developing countries. Current mosquito control strategies depend heavily on insecticides but mosquito populations in various disease-endemic countries are developing resistance (Hemingway and Ranson 2000). Because pyrethroids are the only insecticide class that has World Health Organisation Pesticides Evaluation Scheme (WHOPES) approval for use on insecticide-treated nets, pyrethroid resistance can seriously hamper vector control activities. Not only does insecticide resistance reduce the capacity to repel and kill mosquitoes, there is also evidence that insecticides can select for certain behaviourally resistant traits, such as earlier mosquito feeding times and earlier exiting from houses with treated nets (Mathenge et al. 2001, Pates and Curtis 2005). Furthermore, resistance to some insecticides can confer cross-resistance to other insecticides, notably the organochlorine DDT (Chandre et al. 1999b, Brooke et al. 2001, Brengues et al. 2003, Enayati et al. 2003). There is, therefore, an urgent need for alternative tools or strategies that can effectively control insecticide-resistant mosquito populations.

At present biocontrol and biopesticide agents are only operational against mosquito larvae and pupae (Kay et al. 2002, Mittal 2003, Howard et al. 2007, Howard et al. 2009) (**Chapters 3 & 6**). However, it is the longevity of the adult

mosquito that has the greatest impact on the vectorial capacity, and hence transmission intensity, of a mosquito population (MacDonald 1957). Biocontrol agents that target the adult mosquitoes, and to which resistance cannot readily develop, would be useful tools for mosquito control.

The hyphomycetous entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* have been used to target pest insects for over a century (Lord 2005), and have recently been evaluated for mosquito control purposes (see Table 7.1). These fungi infect mosquitoes through direct contact with the cuticle. The fungal conidia penetrate the mosquito cuticle and grow into the haemocoel where they produce a blend of organic compounds, causing internal mechanical damage, nutrient depletion and death (Gillespie and Clayton 1989). Lethal effects start to occur three to four days after infection (Scholte et al. 2003b, Scholte et al. 2003a, Achonduh and Tondje 2008, Farenhorst et al. 2008). These entomopathogenic fungi are effective at killing both insecticide-resistant and insecticide-susceptible mosquito populations (Farenhorst et al. 2009, Kikankie et al. 2010). Furthermore, *M. anisopliae* and *B. bassiana* kill mosquitoes in a slower manner than insecticides kill insecticide-susceptible mosquito populations (Scholte et al. 2003a, Kamala Kannan et al. 2008, Mnyone et al. 2009a). To prevent the evolution of resistance it is important to let organisms reproduce before they are killed, to allow more than just the individuals with resistance/tolerance genes to contribute to the next generation. It is therefore thought that resistance to fungi will not evolve readily and that they have the possibility to be “evolution-proof” (Thomas and Read 2007, Read et al. 2009). This late acting approach is possible in malaria control where the extrinsic incubation period (EIP) of the parasite is usually three to four gonotrophic cycle lengths (depending on temperature and female susceptibility to infection with *Plasmodium*). Ideally the fungi would kill the mosquito after reproduction had occurred but before she can transmit the malaria parasite.

Previous studies have used many different combinations of formulation/substrate (Table 7.1) to demonstrate the effectiveness of entomopathogenic fungi to infect

and kill mosquitoes. However, many of the application methods previously used cannot be deployed easily in the field, either for small-scale tests or for operational vector control. Because fungal spores are biological entities that are affected by the application (formulation/substrate) methods used, it is important to test potential methods that can be used in the field. Many traditional rural African houses are built with open eaves to help air flow within the house. Trials in The Gambia and São Tomé have shown that eaves are important house entry points for *Anopheles gambiae* Giles *s.l.* (Charlwood et al. 2003, Njie et al. 2009). Rural African houses also tend to have open windows through which mosquitoes can enter. Eave curtains and insecticide-treated curtains have proven effective at decreasing the numbers of indoor-resting mosquitoes (Majori et al. 1987) and reducing child mortality (Diallo et al. 2004). Curtains have a smaller surface area than bednets, do not come into close contact with humans and would be hung where mosquitoes enter houses. Application of fungal spores onto curtains may, therefore, be an interesting application method for mosquito control in the field.

There were two objectives of this study; the first was to compare the fungal susceptibility of an insecticide-resistant and insecticide-susceptible strain of *Anopheles gambiae s.s.*. The second objective was to test a potential application method that could be used in the field. Therefore, *M. anisopliae* and *B. bassiana* conidia were separately suspended in mineral oil and these suspensions were separately applied onto white polyester netting. We then used tube bioassays to test the potential of these nets to infect and kill *An. gambiae s.s.* SKK (an insecticide-susceptible strain) and *An. gambiae s.s.* VKPER (an insecticide-resistant strain) mosquitoes at different time points after the nets had been treated with fungal conidia. In addition, fungal viability after application onto the polyester nets was measured.

Table 7.1. Different formulation/substrate application methods used to infect adult malaria vector mosquitoes with the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in previous studies

Fungus	Formulation	Substrate	Mosquito species	Lab or field	Reference
B. bassiana	Dry conidia		*An. albimanus*	Laboratory	(Clark et al. 1968)
B. bassiana	Dry conidia	Agar plate	*An. gambiae s.s.*	Laboratory	(Scholte et al. 2003a)
B. bassiana	Dry conidia	Plastic tube	*An. gambiae s.s.,*	Laboratory	(Farenhorst et al. 2009)
			An. funestus, An. arabiensis		(Kikankie et al. 2010)
B. bassiana	Dry conidia	Tissue paper	*An. gambiae s.s.*	Laboratory	(Achonduh and Tondje 2008)
B. bassiana	Ondina oil	Cardboard	*An. gambiae s.s.*	Laboratory	(Farenhorst and Knols 2010)
B. bassiana	Ondina oil	Paper and net	*An. gambiae s.s.*	Laboratory	(Mnyone et al. 2009b)
B. bassiana	Ondina/ShellSol	Cage mesh	*An. stephensi*	Laboratory	(Blanford et al. 2005 & 2009)
B. bassiana	Ondina/ShellSol	Cardboard pot	*An. stephensi*	Laboratory	(Blanford et al. 2005)
B. bassiana	Ondina/ShellSol	Direct application	*An. stephensi*	Laboratory	(Blanford et al. 2005)
B. bassiana	ShellSol T	Cardboard	*An. gambiae s.s.*	Laboratory	(Farenhorst and Knols 2010)
B. bassiana	ShellSol T	Proofing paper	*An. gambiae s.s.*	Laboratory	(Farenhorst and Knols 2010)
M. anisopliae	Coconut oil	Filter paper	*An. stephensi*	Laboratory	(Kamala Kannan et al. 2008)

M. anisopliae	Dry conidia		*An. stephensi*	Laboratory	(Kamala Kannan et al. 2008)
M. anisopliae	Dry conidia	Agar plate	*An. gambiae s.s.*	Laboratory	(Scholte et al. 2003a)
M. anisopliae	Dry conidia	Plastic tube	*An. gambiae s.s.*	Laboratory	(Scholte et al. 2003b)
M. anisopliae	Enerpar oil	Proofing paper	*An. gam s.s., An. arab*	Laboratory	(Mnyone et al. 2009a)
M. anisopliae	Enerpar/Ondina	Black cotton	*An. arabiensis*	Field	(Lwetoijera et al. 2010)
M. anisopliae	Ondina oil	Paper and net	*An. gambiae s.s.*	Laboratory	(Mnyone et al. 2009b)
M. anisopliae	Ondina oil	Cardboard	*An. gambiae s.s.*	Laboratory	(Farenhorst and Knols 2010)
M. anisopliae	Ondina oil	Clay pot	*An. gam s.s., An. fun*	Laboratory	(Farenhorst et al. 2008)
M. anisopliae	Ondina/ShellSol	Cardboard pot	*An. stephensi*	Laboratory	(Blanford et al. 2005)
M. anisopliae	Ondina/ShellSol	Cage mesh	*An. stephensi*	Laboratory	(Blanford et al. 2009)
M. anisopliae	ShellSol T	Cardboard	*An. gambiae s.s.*	Laboratory	(Farenhorst and Knols 2010)
M. anisopliae	ShellSol T	Proofing paper	*An. gambiae s.s.*	Laboratory	(Farenhorst and Knols 2010)
M. anisopliae	Sunflower oil	Cotton netting	*An. gambiae s.s.*	Laboratory	(Scholte 2004)
M. anisopliae	Sunflower oil	Filter paper	*An. gambiae s.s.*	Laboratory	(Scholte et al. 2003b)
M. anisopliae	Vegetable oil	Black cotton	*An. gambiae s.l.*	Field	(Scholte et al. 2005)
M. anisopliae	Vegetable oil	Mud walls	*An. gambiae s.s.*	Field	(Scholte 2004)

7.3 Materials and Methods

7.3.1 Mosquitoes

The two mosquito strains used in the bioassays were *An. gambiae s.s.* VKPER and *An. gambiae s.s.* SKK. The SKK strain is an insecticide-susceptible strain originating from Suakoko, Liberia and maintained as a laboratory colony at Wageningen University, The Netherlands, since 1989. The VKPER strain is a pyrethroid-resistant strain that was initially collected from the Kou Valley, Burkina Faso and then selected repeatedly to fix the pyrethroid knockdown resistance (*kdr*) gene. This gene causes target site insensitivity (Enayati et al. 2003) and was first reported in West African mosquitoes in the early 1990s (Martinez-Torres et al. 1998). The VKPER strain has been maintained as a colony at the Centre de Recherche Entomologique de Cotonou (CREC) in Benin, West Africa, for several years. Eggs from this colony were shipped to Wageningen University, and a colony was started.

Both mosquito strains were subject to standard rearing procedures using tap water in plastic trays (10 x 25 x 8 cm) and fed with 'Tetramin ®' fish food daily. Pupae were selected daily and adults were held in standard 30 x 30 x 30 cm gauze-covered cages and fed on a 6% glucose solution *ad libitum*. The larval trays and adult cages were kept in climate chambers held at 27 ± 1°C, 80 ± 10% RH and a 12:12 hr L:D photoperiod.

7.3.2 Fungi

We studied the effects of two species of entomopathogenic fungi. *Metarhizium anisopliae* var. *anisopliae* (Metsch.) Sorokin isolate ICIPE-30 was produced at Wageningen University, The Netherlands, using solid-state fermentation in aerated packed bed systems with glucose-impregnated hemp as a growth substrate. *Beauveria bassiana* (Balsamo) Vuillemin IMI391510 was grown in the laboratory of

Penn State University, USA, by initially growing the fungus in a liquid medium and then inoculating autoclaved barley flakes in mushroom spawn bags.

Fungal conidia were dried at ambient temperature (<5% RH) and stored in the refrigerator until use. Dry conidia of *M. anisopliae* and *B. bassiana* were separately suspended in the synthetic isoparaffinic hydrocarbon solvent ShellSol T ™ (Shell, The Netherlands). ShellSol T was selected because the delivery system of fungal conidia suspended in this solvent has been shown to be significantly more virulent to *An. gambiae s.s.* mosquitoes when compared to conidia suspended in other oils (Farenhorst and Knols 2010). A Bürker-Türk haemocyte counter and light microscope (at x400) were used to determine accurate conidial concentrations per ml ShellSol T. Fresh suspensions were made for each experimental replicate.

7.3.3 Net treatment

The netting used was made of white 100% multifilament 150 denier warp-knitted polyester fibres with a mesh size of 12 holes per cm^2 (Vestergaard Frandsen, Switzerland). Pieces 15 x 25 cm were used and dipped in the conidia/ShellSol T suspensions resulting in treatment densities of 7.2 x 10^{12} conidia per m^2. Control netting was treated with ShellSol T only.

Fungus-treated pieces of netting were held in a climate chamber at Wageningen University under constant conditions of 27 ± 1°C and 65 ± 10% RH, to simulate average climatic conditions of field settings. The viability of fungal conidia (see **section 7.3.5** below) was scored at 1 and 7 days post-treatment, and mosquito bioassays (see **section 7.3.4** below) were run 2 and 7 days post-treatment.

7.3.4 Tube bioassays

Separate pieces of control, *M. anisopliae* or *B. bassiana*-treated netting were

placed into a tube bioassay set up (8 cm diameter x 15 cm high; see Figure 1E in Farenhorst and Knols (2010)) such that the netting covered the inside of the tube. These were stored in a climate chamber at Wageningen University at 27 ± 1°C and 65 ± 10% RH until testing. Tests were carried out in the climate chamber on day 2 and day 7 after net treatment.

For the bioassays, the tubes were sealed at both ends with cling film, a surface that mosquitoes do not like resting on. Twenty-five 3-5 day old non-blood fed female *An. gambiae* VKPER or SKK strain mosquitoes were introduced into each tube and exposed to the nets for 1 hr. Four replicates were performed per time point. After the exposure time the mosquitoes were placed into cups and had access to 6% glucose solution *ad libitum*. Every 24 hrs mosquitoes were recorded as being alive if they were still able to fly (World Health Organisation 1998). Mortality was scored until all the fungus-exposed mosquitoes had died.

Dead mosquitoes were removed daily and checked for fungal infection. Cadavers were dipped in 70% ethanol, for external sterilization, and placed onto moist filter paper in Petri dishes that were then sealed with Parafilm and placed into a 27°C incubator in the dark. After three days it was possible to visually score the proportion of mosquitoes showing fungal infection based on the presence of sporulating fungal hyphae (*M. anisopliae* conidia are green (see bottom right photo on the thesis cover); *B. bassiana* conidia are white).

7.3.5 Fungal viability

As a measure for conidial viability, the germination of the conidia on a rich agar medium was counted. Either a drop of the conidial suspension or a 1 cm^2 piece of the treated netting was placed onto Sabouraud Dextrose Agar (SDA) plates. The SDA plates had 0.001% benomyl added so that accurate germination could be recorded; benomyl is a fungicidal compound that restricts the hyphal growth without affecting germination (Milner et al. 1991). These plates were then

incubated at 27°C in the dark and germination was scored 24 hrs later using a light microscope at x400. A conidium was scored as germinated if the germ tube was at least twice the length of the conidium. A minimum of 300 conidia were counted per plate; four replicates of each fungus species/time point were carried out.

7.3.6 Statistical analysis

For the mosquito survival analysis, differences between the control and fungus-exposed mosquito survival were investigated using Cox regression analysis in SPSS 17.0 (SPSS Inc 2008). Significant mosquito strain and fungus species effects were further investigated using Cox regression. Mortality rates were given as Hazard Ratios (HR), which give the average daily risk of dying. Chi-square tests were carried out to investigate the difference between the fungal viability in suspension and on treated nets using SAS 9.1 (SAS Institute Inc. 2004). Statistical analyses were carried out at the 5% significance level.

7.4 Results

7.4.1 Tube bioassays

Both *M. anisopliae* and *B. bassiana* were pathogenic to both strains of *An. gambiae s.s.*, with significantly increased mortality in all fungus-exposed/mosquito strain combinations (Table 7.2). Survival curves for all fungus-infected mosquitoes were significantly different from the respective controls for the mosquitoes exposed two days post net treatment and those exposed seven days after net treatment (Figure 7.1). Furthermore, *B. bassiana* was significantly more pathogenic than *M. anisopliae* both for SKK (day 2 HR=3.47, $p<0.0001$; day 7 HR=2.84, $p<0.0001$) and VKPER (day 2 HR=1.89, $p<0.0001$; day 7 HR=1.45, $p<0.05$).

There was no significant difference between the control VKPER and control SKK

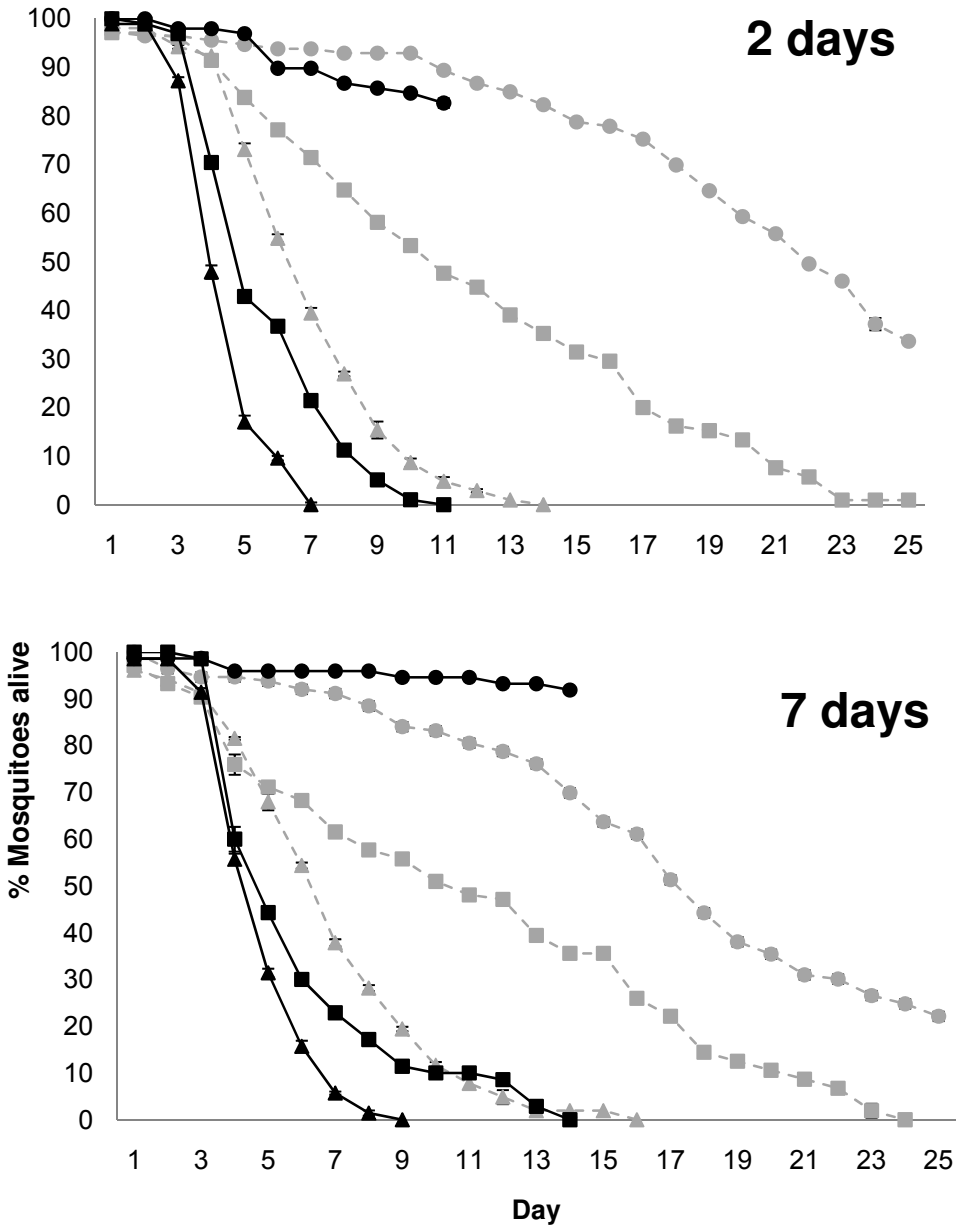

Figure 7.1. Effect of entomopathogenic fungal infection on mean cumulative proportional survival (±SE) of *Anopheles gambiae s.s.* SKK (dashed grey) and *An. gambiae s.s.* VKPER (solid black) mosquitoes after exposure to *Metarhizium anisopliae*-treated (squares), *Beauveria bassiana*-treated (triangles) or control (circles) netting 2 (top) or 7 (bottom) days after net treatment

Table 7.2. Survival analysis of two strains of *Anopheles gambiae s.s.* exposed to two species of entomopathogenic fungi; data show Cox regression Hazard Ratio (HR) outcomes (95% CI), statistical p-values are relative to the relevant control

Fungus	Mosquito Strain	2 days after treatment HR (95% CI)	p	7 days after treatment HR (95% CI)	p
M. anisopliae	SKK	3.18 (2.31, 4.37)	<0.0001	2.60 (1.94, 3.48)	<0.0001
	VKPER	17.10 (9.68, 30.20)	<0.0001	29.94 (12.72, 70.46)	<0.0001
B. bassiana	SKK	11.01 (7.43, 16.32)	<0.0001	7.38 (5.21, 10.45)	<0.0001
	VKPER	32.25 (17.63, 59.02)	<0.0001	43.52 (18.02, 105.11)	<0.0001

SKK = the insecticide-susceptible *An. gambiae s.s.* SKK strain; VKPER = the insecticide-resistant *An. gambiae s.s.* VKPER strain

mortalities (HR=1.63, p=0.053). However, the insecticide-resistant mosquito strain VKPER was significantly more susceptible to fungal infection when compared to the SKK strain after being exposed to the two (*M. anisopliae* HR=4.46, p<0.0001; *B. bassiana* HR=3.59, p<0.0001) and seven (*M. anisopliae* HR=2.54, p<0.0001; *B. bassiana* HR=2.33, p<0.0001) day old net treatments. The number of days since the fungal treatments were applied to the nets caused no significant differences in the mortality of either the SKK (HR=1.02, p=0.85) or VKPER (HR=0.83, p=0.09) mosquitoes. This indicates that despite the significant drop in fungal viability (see **section 7.4.2**), the efficacy of the fungal conidia in terms of mosquito pathogenicity was equally high seven days after net application.

For both VKPER and SKK mosquitoes, >80% of the dead mosquitoes that were exposed to the fungus-treated netting showed evidence of fungal infection in the form of sporulation. Sporulation rates by themselves do not equate to fungal infection because sporulation varies with many things including fungal dose and

virulence of fungal isolate, age of the mosquito and presence of microbial competitors. Although not a perfect indicator for fungal infection, the sporulation of the *M. anisopliae* exposed mosquitoes could be of interest because the viability of the *M. anisopliae* used was so low. For the VKPER mosquitoes that were exposed to the 2 day old *M. anisopliae* treated netting (which had a viability of 13% the day before the bioassay), 82% (80/98) of the mosquitoes showed fungal sporulation. For the mosquitoes exposed to the seven day old *M. anisopliae* net (where the viability was 2%), 84% (59/70) of the mosquitoes showed infection. This was not significantly different from the numbers infected on day 2 (χ^2=0.02, df=1, p=0.65) despite the significant decrease in the viability of the spores on the netting.

7.4.2 Fungal viability

The viabilities, expressed as the germination rate of fungal conidia, of *B. bassiana* and *M. anisopliae* in the ShellSol T suspensions were 77% and 36% respectively. When the treated polyester net was kept in a climate chamber held at 27 ± 1°C, 65 ± 10% RH for one day, the viabilities of *B. bassiana* and *M. anisopliae* were 71% and 13% respectively. These viabilities had both dropped significantly (*B. bassiana* χ^2=5.21, df=1, p<0.03; *M. anisopliae* χ^2=192.9, df=1, p<0.0001) when compared to the viabilities in suspension. The viabilities of the two fungal species after seven days in a climate chamber were 62% and 2% respectively, for *B. bassiana* and *M. anisopliae.* On top of the significant drop in viability one day after fungal spore application, seven days after net treatment there were significant losses in viability when compared to the day 1 viabilities for both fungal species (*B. bassiana* χ^2=50.9, df=1, p<0.0001; *M. anisopliae* χ^2=215.5, df=1, p<0.0001).

7.5 Discussion

For both species of fungus tested, the insecticide-resistant *An. gambiae s.s.*

VKPER strain was significantly more susceptible to fungal infection than the insecticide-susceptible *An. gambiae s.s.* SKK strain. The risk of dying was around 2-4 times higher for VKPER depending on fungal species and age of treatment on the net. A previous study used colony and wild F1 *An. arabiensis* mosquitoes that were exposed to dry conidia of *B. bassiana*. They found no significant differences between the fungal susceptibility of the insecticide-resistant or insecticide-susceptible strains (Kikankie et al. 2010). Another study using dry conidia looked at various *Anopheles* species with various types of insecticide resistance and also found no differences in fungal susceptibility between the insecticide-susceptible and insecticide-resistant strains (Farenhorst et al. 2009). The main difference between our and these previous studies is that in our study, mosquitoes were exposed to ShellSol T formulated conidia for 1 hr, whereas the two studies mentioned above exposed mosquitoes to dry conidia for 24 hrs (Farenhorst et al. 2009, Kikankie et al. 2010). Dry conidia have been shown to kill mosquitoes faster than oil formulated conidia (Scholte et al. 2003b). It is therefore likely that the studies using the 24 hr exposure to dry conidia, whilst good for proving any fundamental principles requiring high fungal infection, caused the mosquitoes to receive such high doses of fungal infection that any subtle strain effects could not be detected.

Beauveria bassiana was significantly more virulent than *M. anisopliae* for both mosquito strains. However, it is likely that the difference in virulence is linked to the differing viabilities of the *B. bassiana* and *M. anisopliae* on the treated nets used in this study, as this would lead to lower doses being received by the *M. anisopliae*-exposed mosquitoes when compared to the mosquitoes exposed to *B. bassiana.* It is possible that batches of *M. anisopliae* with a higher viability would have similar results to *B. bassiana* because most other studies involving adult mosquitoes that have used these two fungal species have found no differences in their virulence. Blanford et al. (2005) tested a range of oil-formulated fungal isolates of *B. bassiana* and *M. anisopliae* against *An. stephensi* mosquitoes. One *M. anisopliae* isolate used did not prove virulent to mosquitoes, whilst the other had the same virulence

as the *B. bassiana* isolates (Blanford et al. 2005). Similarly, a study examining different application methods found similar virulence levels for oil-formulated *M. anisopliae* and *B. bassiana* when applied to both proofing paper and cardboard, and when different doses of each fungus were applied to proofing paper (Farenhorst and Knols 2010). When dry conidia were used, Scholte et al. (2003a) found that *M. anisopliae* was significantly more virulent to mosquitoes than *B. bassiana* after a three day exposure, although it is unclear what the respective viabilities of the conidia were. Another study using dry conidia found that the virulence of *M. anisopliae* and *B. bassiana* were similar for a range of mosquito species and strains (Farenhorst et al. 2009).

Broadly speaking, previous fungal studies in the laboratory have used application methods that fall into three categories; dry conidia, using paper as a substrate and using substrates that can directly be used in the field. Of the latter type, studies have been carried out using mosquito cage mesh (Blanford et al. 2005, Blanford et al. 2009), clay pots (Farenhorst et al. 2008) and cotton netting (Scholte 2004). In addition to these laboratory studies, field studies in Tanzania have used black cotton cloths (Scholte et al. 2005, Lwetoijera et al. 2010) and direct application onto a mud wall (Scholte 2004). Of these studies, the fungal viabilities after application onto the substrates were measured for the cotton netting in the laboratory (Scholte 2004) and the black cotton cloths used in the field (Scholte et al. 2005). In the laboratory, the cotton netting was kept in aluminium foil in the same climate chambers as used in our study, and the viabilities of *M. anisopliae* were 100% in suspension, 94% one day after net treatment and 82% one week after net treatment (Scholte 2004). For the black cotton sheets used in Tanzania, the *M. anisopliae* viability decreased from 96% in suspension to 95% one day after sheet impregnation and 83% after a week (Scholte et al. 2005). These one day and one week drops in viability on cotton were much less than observed in this study on polyester netting with *M. anisopliae,* suggesting cotton may be a more suitable substrate for the application of entomopathogenic fungi. Although the *M. anisopliae* we used was of poor quality, with viability in suspension just 36%, the differences

between fungal viability on cotton and the viability on polyester could also be explained by inherent differences between cotton and polyester netting. Because polyester is a synthetic material, certain chemicals used in its manufacture could be harmful to the fungal conidia. Unfortunately, due to the different conidial viabilities, doses, exposure times and formulations used for this study and the cotton netting laboratory trial (Scholte 2004) it is not possible to directly compare the relative effect of each type of netting/fungus application method at killing mosquitoes in the laboratory.

When looking at the viability data it appears that the polyester netting/ShellSol T application method would not be a very suitable method for the delivery of viable entomopathogenic fungal spores for mosquito control. However, the virulence data examining the direct effect on mosquito mortality tells a different story. Regardless of the time since net treatment with fungi, both fungal species caused significantly increased mortality to both mosquito strains used. The viability of the *M. anisopliae*-treated nets was just 2% seven days after net treatment. However, the effectiveness of the fungal treatment at killing mosquitoes did not significantly deteriorate during the length of the trial and high infectivity rates were observed. The differences between the viability and virulence results may be due to the differing abilities of the fungal conidia to germinate on mosquito cuticles and benomyl-enriched agar. Whilst benomyl has been shown to not adversely affect the germination of *M. anisopliae* spores when compared to their germination in liquid medium (Milner et al. 1991), it would be no surprise that such a difference occurs because benomyl is a fungicide and insects are the natural hosts for these fungi.

It is thought that the slow kill speed of entomopathogenic fungi could lead to them being evolution-proof against resistance (Read et al. 2009). This is because any resistance-related genes would be diluted by the genes of susceptible individuals passed onto the next generation before they have succumbed to the fungal infection (Read et al. 2009). For this to be an ethically acceptable strategy for malaria control, the fungi should kill the mosquitoes before the parasite has

completed its EIP inside the mosquito. The EIP of malaria parasites can be calculated using the equation:

$$N \text{ (days)} = 111/(T - t_{min})$$

where T is the mean temperature and t_{min} is taken as 16°C (Detinova 1962). Our experiments were carried out at 27°C; at this temperature the EIP would be 10 days. If entomopathogenic fungi are used on window curtains or bednets, thus targeting host seeking mosquitoes, then a valid assumption would be that a mosquito acquires both fungal and malaria infections at the same time. Given an EIP of 10 days at our experimental temperature, our results show that for the VKPER strain mosquitoes, all mosquitoes would have been killed by *B. bassiana* by this time, and >90% by *M. anisopliae*. In other words, very few fungus-infected VKPER mosquitoes would have survived long enough to transmit malaria. For the less susceptible SKK strain, *B. bassiana* would have killed 90% and *M. anisopliae* just 50% of the mosquitoes by the time the mosquitoes became infectious with malaria. This slower speed of kill found with *M. anisopliae* infected SKK could allow more malaria transmission to occur, but it will also allow more mosquito reproduction, and thus less chance of resistance to fungal infection developing.

7.6 Conclusions

We show for the first time that insecticide-resistant *An. gambiae s.s.* VKPER are significantly more susceptible to both fungal species when compared to the insecticide-susceptible *An. gambiae s.s.* SKK. This indicates that entomopathogenic fungi could be used in resistance management and integrated vector management programmes to target insecticide-resistant mosquitoes, possibly leading to the conservation of insecticide-susceptible genes in a mosquito population. Field trials over a longer trial period need to be carried out to see if wild insecticide-resistant mosquitoes are as susceptible as the colony strain used in this

trial.

This is the first published study to treat polyester netting with fungal spores. Although fungal viability significantly decreased when applied to polyester netting, the effectiveness of the fungal treatment at killing mosquitoes did not significantly deteriorate during the length of the trial. Following this laboratory trial, studies should be carried out to determine whether polyester netting would be an effective application method for entomopathogenic fungi in the field.

7.7 Acknowledgements

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Chapter 8

The entomopathogenic fungus *Beauveria bassiana* reduces blood feeding in wild insecticide-resistant mosquitoes in Benin, West Africa

Annabel F.V. Howard, Raphael N'Guessan, Constantianus Koenraadt, Alex Asidi, Marit Farenhorst, Martin Akogbéto, Matthew Thomas, Bart Knols, Willem Takken

Submitted in a slightly modified form

8.1 Abstract

Mosquito-borne diseases are still a major health risk in many developing countries, and the emergence of multi-insecticide-resistant mosquitoes is threatening the future of vector control. Therefore, new tools that can manage resistant mosquitoes are required. Laboratory studies show that entomopathogenic fungi can kill insecticide-resistant malaria vectors but this needs to be verified in the field. The present study investigated whether these fungi will survive under field conditions and be effective against wild multi-insecticide-resistant West African mosquitoes. The entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* were separately applied to white polyester netting and used in combination with either a permethrin or untreated bednet in an experimental hut trial to examine the effects of the two species of fungi on the survival and behaviour of wild mosquitoes. In total, 1125 female mosquitoes were collected during the hut trial, mainly *Culex quinquefasciatus* Say. Unfortunately, not enough wild *Anopheles gambiae* Giles were collected to allow the effect the fungi may have on this malaria vector to be analysed. None of the treatment combinations caused significantly increased mortality of *Cx. quinquefasciatus* when compared to the control hut. The only significant behaviour modification found was a reduction in blood feeding by *Cx. quinquefasciatus,* caused by the permethrin and *B. bassiana* treatments, although no additive effect was seen in the *B. bassiana* and permethrin combination treatment. This is the first time that an entomopathogenic fungus has been shown to reduce blood feeding of wild mosquitoes. This behaviour modification indicates that *B. bassiana* could potentially be a new mosquito control tool effective at reducing disease transmission, although further field work specifically targeting malaria vectors should be carried out to verify this. In addition to the *Culex* findings, laboratory tests and field bioassays examining the behaviour and mortality of an insecticide-resistant strain of the malaria vector *An. gambiae s.s.* in response to fungal infection showed significant mortality in the field, and no behavioural deterrence. Conidial viability decreased under field conditions.

8.2 Introduction

Current mosquito control for the prevention of malaria and other vector-borne diseases relies heavily on pyrethroid insecticides, most notably through the use of insecticide-treated nets (ITN) and indoor residual spraying (IRS) (World Health Organisation 2009). Unfortunately, the emergence of insecticide resistance in some geographical areas is threatening vector control efforts (N'Guessan et al. 2007). It is widely accepted that the emergence of insecticide resistance in mosquitoes in Benin, as in many other areas of the world, was due to heavy pesticide use in agriculture (Lines 1988, Diabate et al. 2002, Akogbeto et al. 2005, Corbel et al. 2007, Yadouleton et al. 2009). However, the impact of this resistance is increasingly affecting the public health sector as well. It is therefore important to search for alternative tools that can be used to control insecticide-resistant mosquitoes.

The entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* can be used to target a wide range of insects (Lord 2005, Thomas and Read 2007) including adult mosquitoes (Scholte et al. 2003a, Achonduh and Tondje 2008). The conidia of these fungi, once germinated, directly penetrate the mosquito cuticle. Once inside the mosquito haemocoel, the fungi produce compounds and eventually kill the mosquitoes by a combination of nutrient depletion and internal mechanical damage (Gillespie and Clayton 1989). According to previous research, this starts leading to insect death approximately three-to-four days after infection (Scholte et al. 2003b, Scholte et al. 2003a, Farenhorst et al. 2008). This slow kill time is in contrast to fast acting insecticides currently in use. However, because the malaria parasite takes >10 days to develop within the mosquito, even a relatively modest speed of kill can prevent malaria transmission, as long as coverage (i.e. probability of fungal infection per feeding cycle) is high (Hancock 2009, Read et al. 2009). Moreover, previous studies have revealed reductions in mosquito feeding propensity and fecundity (Scholte et al. 2006) due to fungal infection, and *B. bassiana* was shown to limit the development of malaria parasites in the mosquito

(Blanford et al. 2005). In addition, slow speed of kill potentially allows fungus-infected mosquitoes to attain some of their life-time reproductive output, which could reduce selection pressure for resistance. Accordingly, in order to determine overall transmission blocking (and indeed, overall fitness costs of infection) it is important to evaluate not only the mortality rate but also sub- and pre-lethal consequences of infection.

Metarhizium anisopliae and *B. bassiana* are effective at infecting and killing a range of insecticide-susceptible mosquitoes including many *Anopheles* (Scholte et al. 2004b, Kamala Kannan et al. 2008, Mnyone et al. 2009a), *Culex* (Scholte et al. 2003b, Scholte et al. 2004b) and *Aedes* (Scholte et al. 2007, de Paula et al. 2008) mosquito species. In addition, these fungi can cause significant mortality to insecticide-resistant *Anopheles* mosquitoes in the laboratory (Farenhorst et al. 2009, Kikankie et al. 2010), with insecticide-resistant mosquitoes being significantly more susceptible to fungal infection when compared to insecticide-susceptible mosquitoes (Howard et al. 2010) (**Chapter 7**), leading to interesting possibilities with population dynamics and the conservation of insecticide-susceptible genes. Despite the encouraging results from the laboratory, only two studies have been published using these entomopathogenic fungi against mosquitoes in the field, and neither of these studies targeted insecticide-resistant mosquitoes (Scholte et al. 2005, Lwetoijera et al. 2010). One study used extra-domiciliary odour-baited traps to target wild *Anopheles arabiensis* with *M. anisopliae*-treated black cotton eave baffles and panels (Lwetoijera et al. 2010). Another study in rural Tanzania impregnated cotton sheets with *M. anisopliae* that were suspended from the ceilings of houses so that resting mosquitoes would come into contact with the fungus (Scholte et al. 2005). Both of these field studies showed that fungal infections significantly shortened the life span of infected mosquitoes when compared to uninfected mosquitoes (Scholte et al. 2005, Lwetoijera et al. 2010), but neither study examined behavioural effects such as blood feeding.

Because entomopathogenic fungi are themselves living organisms it is important to

test whether they will survive and be effective in field conditions where the temperature and humidity fluctuate. Also, due to proposed future application methods, it is important to test them alone and in the presence of existing control tools such as ITNs to monitor any potential additive or synergistic effects (Hancock 2009). In addition, the increasing insecticide resistance in mosquitoes makes it vital to test entomopathogenic fungi against wild insecticide-resistant mosquitoes, especially given the recent findings that insecticide-resistant mosquitoes are more susceptible to fungal infection than insecticide-susceptible mosquitoes (Howard et al. 2010) (**Chapter 7**), and that fungal infection can restore part of the mosquito's susceptibility to insecticides (Farenhorst et al. 2009).

In this study, an experimental hut trial was conducted in Benin, West Africa, to assess whether wild multi-insecticide-resistant mosquitoes would be infected by *M. anisopliae* or *B. bassiana* when applied to window netting. These fungal treatments were evaluated in the presence of an untreated or permethrin-treated bednet. We examined mortality, effect on blood feeding and other behaviours such as deterrence and induced exophily. In addition, the virulence of these two species of entomopathogenic fungi towards a laboratory colony of the malaria vector *Anopheles gambiae* Giles *s.s.* was examined under field conditions, and the effect of field exposure on conidial viability was determined.

8.3 Materials and Methods

8.3.1 Mosquitoes

The mosquitoes used in the behaviour experiments in the laboratory, and the cone bioassays in the field, were *An. gambiae s.s.* VKPER strain. This is a pyrethroid-resistant strain that was initially collected from the Valley du Kou in Burkina Faso and then selected repeatedly to fix the *kdr* gene. This gene is linked to knockdown resistance to pyrethroids and DDT, and was first reported in West African

mosquitoes in the early 1990s (Martinez-Torres et al. 1998). The VKPER strain has been maintained as a colony at the Centre de Recherche Entomologique de Cotonou (CREC) in Benin for several years. For the laboratory experiments, eggs from this colony were brought to Wageningen University in The Netherlands and a colony was started. Mosquitoes were subject to standard rearing using tap water in plastic trays (10 x 25 x 8 cm) and fed with 'Tetramin ®' fish food daily. Pupae were selected daily and adults were held in standard 30 x 30 x 30 cm cages and fed on a 6% glucose solution *ad libitum*. The larval trays and adult cages were kept in climate chambers held at 27°C (±1), 80% RH (±10) and a 12:12 hr L:D photoperiod.

At the field site (described in **section 8.3.5** below) in Benin, West Africa, the wild *An. gambiae* population has been shown to be 100% *An. gambiae s.s.* Mopti cytotype (Corbel et al. 2007). *Culex quinquefasciatus* Say is also present and both species are resistant to pyrethroids, DDT and dieldrin (Corbel et al. 2007, N'Guessan et al. 2007, Irish et al. 2008, Yadouleton et al. 2010). Resistance mechanisms involve the *kdr* gene mutation, mixed function oxidase (MFO) and esterase levels that are higher than in reference susceptible strains (Corbel et al. 2007). In addition, *Cx. quinquefasciatus* is resistant to carbosulfan and has elevated glutathione-*S*-transferase (GST) activity (Corbel et al. 2007).

8.3.2 Fungi

We examined the effect of two fungal species. *Metarhizium anisopliae* var. *anisopliae* (Metsch.) Sorokin isolate ICIPE-30 was produced using solid state fermentation with glucose-impregnated hemp in 200 ml aerated packed tubes at Wageningen University, The Netherlands. *Beauveria bassiana* (Balsamo) Vuillemin IMI 391510 was produced by initially growing the fungus in a liquid medium and then inoculating autoclaved barley flakes in mushroom spawn bags at Penn State University, USA.

After being dried at ambient temperature and then stored in the refrigerator, dry conidia of *M. anisopliae* and *B. bassiana* were separately suspended in the synthetic isoparaffinic hydrocarbon solvent ShellSol T ™ (Shell, The Netherlands). ShellSol T was selected because the delivery system of fungal conidia suspended in this solvent has been shown to be significantly more virulent to *An. gambiae s.s.* mosquitoes when compared to conidia suspended in other oils (Farenhorst and Knols 2010). A Bürker-Türk haemocyte counter and light microscope (at x400) were used to determine accurate conidial concentrations per ml ShellSol T. New suspensions were made for each experimental replicate.

8.3.3 Net treatment with the fungal conidia

The netting used was made of white 100% multifilament 150 denier warp-knitted polyester fibres with 12 holes per cm^2 (Vestergaard Frandsen, Switzerland). This net was used to cover the windows in the experimental hut trials, for the behaviour experiments in the laboratory, and the cone bioassays in the field. In a preliminary study we found that around 50% of mosquitoes would pass the netting when a human host cue was provided on the other side (Hilhorst et al., unpublished data). In an effort to increase the proportion of mosquitoes passing through the netting, small slits were cut into the netting to facilitate mosquito passage (Figure 8.1). Obviously, in a proper control setting we would not advocate damaging the physical integrity of window or eave screens but for this experimental test, it was necessary to allow mosquitoes access to the huts in order to sample them post-exposure. Netting was dipped into the fungal conidia/ShellSol T suspensions resulting in treatment densities of 7.2×10^{12} conidia per m^2. Control netting was treated with ShellSol T only.

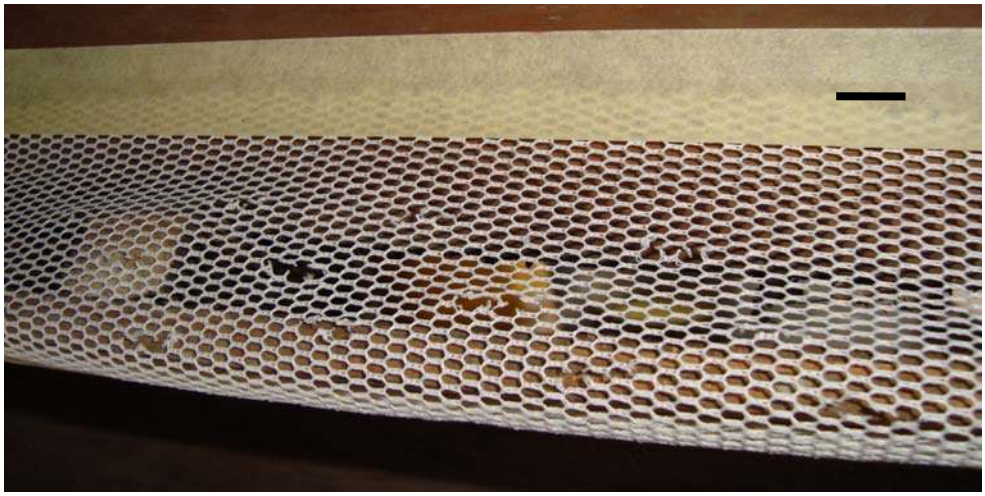

Figure 8.1. A close up of netting attached to the inside of an experimental hut window clearly showing the slits cut to facilitate mosquito passage through the netting; the scale bar (top right) represents 1 cm

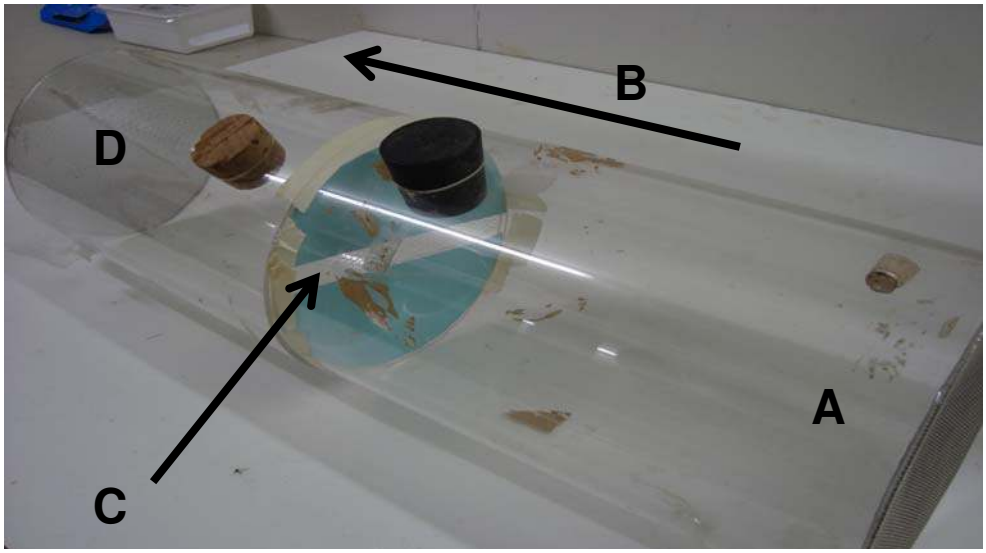

Figure 8.2. The behaviour experiment apparatus used whilst monitoring the movement of mosquitoes through the gap or slit netting in the laboratory. Mosquitoes were introduced into one half of the cylinder (A), they move in the direction indicated (B), crossing the card (C) until they reach the attractive odour sources (not shown but in position (D))

8.3.4 Behaviour experiments in the laboratory

To test the suitability of fungus-treated nets for infecting hut-entering mosquitoes, and to confirm that mosquitoes would be able to pass through the netting, behavioural assays were conducted in the laboratory at Wageningen University. The experimental set-up (Figure 8.2) contained a transparent plastic cylinder (15 cm diameter x 50 cm length) with a separating piece of cardboard in the centre (C in Figure 8.2). A 1 cm slit was made lengthways in the centre part of the cardboard, representing the gaps of the windows in the experimental huts. The ends of the cylinder were sealed with wire netting to allow air to pass through. At one end, a heating element set between 33.5°C and 34.5°C, humid air and a worn nylon sock (not shown but in position D in Figure 8.2) were used to entice mosquitoes released at the other end of the cylinder (A in Figure 8.2) to pass through the 1 cm gap. A small amount of suction at the opposite end was used to move the odour through the cylinder. The test was carried out under a red light and started during the night so that the mosquitoes were more likely to initiate host seeking.

In the first set of experiments in one of the cylinders, the 1 cm gap in the cardboard was left clear, while in the second cylinder untreated white polyester netting was placed over the gap. Six-to-nine day old non-blood fed female *An. gambiae s.s.* VKPER strain mosquitoes were selected immediately before the test based on a response to a human hand; mosquitoes of this age were used because host seeking peaks at 6 days post emergence (Takken et al. 1998). Twenty-five mosquitoes were placed into each tube at a time, such that they had to pass the gap or net to contact the heat and odour source. The test was run for 30 minutes; four replicates were carried out.

In the second set of experiments both cylinders had the slit-cut netting covering the cardboard gap; one cylinder was the control and in the other, the net had been treated with *B. bassiana* 24 hours before the test began. Fifty 6-9 day old non-blood fed female *An. gambiae s.s.* VKPER mosquitoes were selected per replicate, with two replicates run per cylinder. The tests were run for 1 hour, after which time

all mosquitoes that had passed/not passed the netting were removed and kept in cups and given access to 6% glucose solution *ad libitum*. After five days surviving mosquitoes were killed, dipped in 70% ethanol (to sterilize them externally) and placed onto moist filter paper in Petri dishes. These were then sealed with Parafilm and placed in a 27°C incubator in the dark. Three days later the proportion of the mosquitoes infected with the fungus was visually scored by checking the presence of sporulation/emerging hyphae. In this way, it was possible to determine the minimum proportion of the mosquitoes that had passed through the netting that had picked up a fungal infection. Similarly, we scored how many mosquitoes had contacted the netting, picked up an infection but had not passed through the net.

8.3.5 Field study location and experimental hut design

The experimental hut study was undertaken in Ladji village (6°23'23N, 2°25'56E) on the shore of Lake Nokoué in the northern outskirts of Cotonou, in Benin, West Africa. Concrete experimental huts have been built within this village (Figure 8.3) so that they more accurately represent the village dynamics with respect to mosquito house entry. These huts were of the typical West African design with corrugated iron roofs that do not have eaves (for a schematic representation see Hougard et al. (2007)). The ceilings of the huts were thick polyethylene sheeting. Mosquitoes can only enter the huts through four windows. These windows were 60 cm long and consist of metal funnels that channel mosquitoes into a 2 cm gap. This means that once mosquitoes have entered the hut they are unlikely to leave via the windows. Mosquitoes wanting to leave the huts instead fly towards the large veranda trap which, being partial netting, is lighter than the hut interior. The huts are protected from ants by a water moat.

Figure 8.3. One of the experimental huts (on the right) used for the trial; these huts are located inside a village and houses can be seen on the left

8.3.6 Pre-intervention mosquito entry

The intention of the experimental hut trial was to use the window gaps of the experimental huts for the application of fungus-impregnated netting to target the entering mosquitoes with fungal spores. Therefore, before the field trial it was necessary to check whether wild mosquitoes would pass through the window netting. Three of the above-described huts had control netting attached to the inside of the funnelled windows such that every mosquito that entered the huts had to pass through the netting. In the other three huts, the windows were left uncovered. The netting and uncovered windows were then rotated between the huts. This preliminary trial was run for 17 nights from the 6th to the 25th April 2009.

8.3.7 Hut treatments

By using treated netting across the opening of the funnel windows of the experimental huts (Figure 8.1) we ensured that all the mosquitoes entering the huts contacted the fungal spores. The fungus-treated netting was treated as described in **section 8.3.3** above. The control window netting was treated with ShellSol T. Netting was treated and attached to the inside of the window openings in the huts on the same day. For each of the six replicates, new pieces of netting were impregnated with freshly made fungal suspensions. The fungus treatment amounted to 0.13 m^2 per hut.

The bednets used were white 100 denier polyester netting (SiamDutch Mosquito Netting Co., Thailand) measuring 2.11 m length x 1.63 m width x 1.84 m high with a total surface area of 17.2 m^2. Each bednet had six holes cut (4 cm x 4 cm) as recommended by the World Health Organisation (WHO) (World Health Organisation 2006) to mimic worn bednets; this allows blood feeding behaviour to be monitored. Three nets were treated with permethrin 25EC (Syngenta, Switzerland) at 500 mg/m^2 and three others were left untreated to serve as controls. Untreated nets were used because we wanted to test the effect of fungus alone and in combination with an insecticide to examine any potential additive or synergistic effects between the fungi and permethrin treatments.

The six treatments (Table 8.1) were randomly allocated between six huts and then rotated weekly using a Latin square design such that each treatment spent one week in each hut. The hut trial was run for 36 nights between 27th April and 6th June 2009. A temperature and humidity gauge was left inside one of the huts for the duration of the trial.

8.3.8 Hut trial procedure

The study received ethical approval from the Ministry of Health, Cotonou, Republic

of Benin, in May 2008 (approval n° 10717/MSP/DG/SGM/DRS). Six adult males from Ladji village were then recruited as sleepers after they had provided their informed consent to participate in the study. Malaria treatment was offered to these sleepers if they developed malaria during the trial. To control for individual attractiveness (Lindsay et al. 1993a), the sleepers rotated between the huts nightly. During the evening the day after a new treatment had been placed in the huts, all the sleepers were individually asked a short series of questions to determine whether they had any health issues associated with the treatments. The questionnaire was carried out over six weeks such that all sleepers were questioned after sleeping under each treatment.

The external window shutters on the huts were opened at 6pm and the sleepers entered the huts at 9pm. At 5am the following morning a curtain was unrolled to separate the veranda from the hut and at this time the sleepers collected all dead and alive mosquitoes using a mouth aspirator. The mosquitoes from the hut, veranda trap and those found inside the bednet were kept in separate cups. The collected mosquitoes were identified to sex/species and the females were recorded as dead/alive and blood fed/unfed. Live mosquitoes were then held in plastic cups, given access to honey solution and mortality was scored every 24 hours (World Health Organisation 1998). For logistical reasons the mosquitoes that arrived in the laboratory alive from the huts were only able to be kept for a maximum of 7 days, after which time they were killed.

While monitoring the impact of the treatments on mosquito survival, a series of behavioural outcomes were also scored. When compared to mosquitoes collected from the control (Table 8.1; CC) hut it was possible to see whether any of the treatment combinations had caused blood feeding inhibition (smaller proportion of blood fed mosquitoes). Furthermore, if a treatment deters mosquitoes from entering the huts then the proportions that were blood fed may underestimate the full personal protective effect. This can be calculated using the following formula:

$$\% \text{ Personal Protection} = 100(B_u - B_t)/B_u$$

where B_u is the total number of blood fed mosquitoes collected from the untreated control hut and B_t is the total number of blood fed mosquitoes collected from the treated hut (World Health Organisation 2006). In addition, any effects on deterrence (fewer mosquitoes entering the huts) and/or induced exophily (more mosquitoes entering the veranda trap) were measured.

8.3.9 Fungal viability in the field

In Cotonou, Benin, pieces of netting were treated with fungal conidia as described in **section 8.3.3** above and kept under ambient field conditions out of direct sunlight in a well ventilated storage shed to the side of the laboratory. Indoor conditions were chosen as it is proposed that entomopathogenic fungi will be used to target host seeking mosquitoes in people's houses (Scholte et al. 2005). The same pieces of netting used for these viability measurements were used for the bioassays as described in **section 8.3.10** below. Pieces of netting that had been held under field conditions in Cotonou, Benin for 2, 4, 7, 10, 13, 16 and 20 days were transported back to Wageningen University to score fungal viability. As a positive control, samples of the conidial suspensions that had been kept in the fridge were also transported back and tested. Forty-eight hours elapsed between removing the samples from the field conditions in Cotonou and putting them on agar plates in Wageningen University.

As a measure for conidial viability, the germination of spores on a rich agar medium was counted. Either a drop of the conidial suspension or 1 cm^2 of the treated netting was placed onto Sabouraud Dextrose Agar (SDA) plates. The SDA plates had 0.001% benomyl added so that accurate germination could be recorded; benomyl is a fungicide that restricts the hyphal growth without affecting germination (Milner et al. 1991). These plates were then incubated at 27°C in the dark and germination was scored 24 hrs later using a light microscope at x400. A conidium was scored as germinated if the germ tube was at least twice the length

of the conidium. A minimum of 300 conidia were counted per plate. To accurately measure the viability of the spores in the ShellSol T suspension, seven replicates were carried out.

8.3.10 Cone bioassays with fungus-treated netting

To test fungal efficacy after application and storage, WHO cone bioassays were carried out in the field 1, 3 and 5 days post net treatment, using nets treated and stored under field conditions as described above. The cones and netting were set up so that mosquitoes had no alternative but to rest with their tarsi on the netting. This was achieved by suspending the treated pieces of netting between pieces of plastic with holes in them such that the plastic kept the cones in place but the holes ensured that the mosquitoes had to rest on the netting.

Ten-to-twelve 2-3 day old non-blood fed *An. gambiae s.s.* VKPER females were introduced into each of the four replicate cones per treatment (control, *M. anisopliae, B. bassiana*). Because there was no previously published record of WHO cone bioassays being used to infect mosquitoes using entomopathogenic fungi applied to netting, we estimated that an exposure time of 2 hours would allow the maximum chance of infection for the one day old fungal treatment, allowing us to examine any drop off in persistence on the nettings treated 3 or 5 days previously.

After the exposure period, mosquitoes were held in cups in the laboratory in Cotonou and given access to honey solution. Mortality was scored every 24 hours. For logistical reasons mosquito mortality could only be monitored up to day seven post exposure.

8.3.11 Statistical analysis

8.3.11.1 Behaviour experiments in the laboratory

Due to differences between the replicates with respect to the initial mosquito responsiveness, the behaviour experiment data were analysed 10 minutes after the first mosquitoes had passed the gap/netting. This is because it was observed that after this time the vast majority of the mosquitoes that were going to respond to the odour had already responded. Data on mosquito passage was analysed using Chi-square tests. Due to the low numbers of mosquitoes that did not become infected by the *B. bassiana* in experiment two, the Fishers exact test was used to analyse the difference in fungal infection rates between the mosquitoes which passed the net compared to those that did not pass.

8.3.11.2 Hut trial data

Blood feeding was analysed using binomial logistic regression. Statistical outcomes were given as Odds Ratios (OR) which gives the ratio of the odds of an event occurring in one group to the odds of it occurring in another group. The survival analyses of the mosquitoes collected from the huts were investigated using Cox regression analysis. Mortality rates were given as Hazard Ratios (HR), which give the average daily risk of dying relative to the control. Hut attractiveness, treatment deterrence, induced exophily and immediate mortality were separately analysed using single factor ANOVA analysis.

8.2.11.3 Fungal viability in the field

Although only a few data points were collected per fungal species, to investigate whether the viability of fungal conidia significantly deteriorated with time simple linear regression analysis was carried out.

8.3.11.4 Cone bioassays with fungus-treated netting

The replicates were not significantly different from each other, so the data were pooled. For survival analysis, differences between the control and fungus-exposed mosquito survival rates were investigated using Cox regression analysis. Significant day and fungal species effects were further investigated using Cox regression. As with the hut trial data, mortality rates were given as Hazard Ratios (HR).

All statistics were carried out in SPSS 17.0 (SPSS Inc 2008) with α at 0.05.

8.4 Results

8.4.1 Behaviour experiments in the laboratory

In the first set of experiments, there was no significant difference between the numbers of *An. gambiae s.s.* VKPER mosquitoes that passed the gap (48/100) compared to those that passed the net with slits cut into it (45/100) (χ^2=0.18, df=1, p=0.67). Similarly, in the second set of experiments there was no significant difference between the number of *An. gambiae s.s.* VKPER passing either the control (59/96) or *B. bassiana* (57/103) treated net (χ^2=0.77, df=1, p=0.38), indicating that this malaria vector is not deterred by the entomopathogenic fungus. Of the mosquitoes that passed the treated net, 98% (56/57) showed infection with *B. bassiana* after death, while 89% (41/46) of mosquitoes that did not pass the netting showed *B. bassiana* infection; this difference was not significant (Fishers exact test; p=0.09).

These results showed that our proposed protocol for the field work, where mosquitoes were expected to pass through screened windows, should allow mosquitoes to enter the huts through the slit netting leading to fungal infection.

8.4.2 Pre-intervention mosquito entry

Over the 17 pre-intervention nights in the experimental huts, 1356 mosquitoes were collected. Of the 1073 females, 86.7% were *Cx. quinquefasciatus* and 13.3% *An. gambiae s.l.*. When compared to the number of mosquitoes entering the huts without the netting, the untreated slit window nets reduced culicine female entry by 29% and anopheline female entry by 64%.

8.4.3 Hut trial data

During the hut trial the temperature and humidity ranges were 25.1 - 36.4°C and 69 - >95%RH respectively inside the huts, with daily means (±SE) of 30.8°C (±0.23) and 84%RH (±1.33). For each week the maximum recorded temperature and humidity was above 34°C and 95% RH respectively. Out of the 216 questions asked to the sleepers during the trial, no adverse effects (such as respiratory difficulties, skin irritation or headaches etc.) due to the fungal treatments were reported.

A total of 1955 mosquitoes were collected in the huts over 36 intervention nights; 1018 *Cx. quinquefasciatus* females, 87 *An. gambiae s.s.* females, 20 *Aedes aegypti* L. females and 830 males of several different genera. Only seven *An. gambiae s.l.* females entered our control (CC) hut during the six-week hut trial. The 64% reduced entry rate calculated during pre-intervention data collection (**section 8.4.2**) indicates that only a predicted 19 *An. gambiae s.l.* would have entered the CC hut if there was no netting on the windows. This would still not have been enough to carry out adequate statistical analysis. Due to the low number of *An. gambiae s.s.* at the time of the study, only *Cx. quinquefasciatus* data were analysed and presented.

Of the 1018 female *Cx. quinquefasciatus* collected during the experimental hut trial, 22.5% (229/1018) had blood fed. The proportion of blood-fed *Cx. quinquefasciatus*

Table 8.1. Experimental hut data showing the effects on mortality and blood feeding in wild multi-insecticide-resistant *Culex quinquefasciatus* mosquitoes caused by fungal and insecticide treatment combinations; significant p-values are in bold

Window treatment	**Bednet treatment**	**Code**	**N**	**Mortality at 7 days % (95% CI)**	**Blood fed % (95% CI)**	**BFI%**	**PP%**	**Blood fed OR (95% CI)**	**p**
Control	Control	CC	207	53.6 (46.8,60.4)	30.4 (24.2,36.7)	-	-	-	-
Control	Permethrin	CP	167	50.9 (43.3,58.2)	17.4 (11.6,23.1)	42.9	19.3	0.53 (0.3,0.9)	**=0.012**
M. anisopliae	Control	MC	177	50.8 (43.3,58.2)	31.6 (24.8,38.5)	-3.9	14.5	1.15 (0.7,1.8)	=0.54
M. anisopliae	Permethrin	MP	127	52.8 (44.1,61.4)	15.0 (8.8,21.2)	50.8	38.7	0.58 (0.3,1.0)	**=0.045**
B. bassiana	Control	BC	168	57.7 (50.3,65.2)	19.0 (13.1,25.0)	37.4	18.8	0.58 (0.4,0.9)	**=0.032**
B. bassiana	Permethrin	BP	172	54.1 (46.6,61.5)	17.4 (11.8,23.1)	42.7	16.9	0.53 (0.3,0.9)	**=0.012**

BFI = Blood Feeding Inhibition; PP = Personal Protection; OR = Odds Ratio

was significantly lower in four treatment combinations when compared to the control treatment (Table 8.1). The level of blood feeding inhibition was similar for all three treatments that incorporated permethrin (CP, MP and BP). Of the fungus-only treatments, only the *B. bassiana* (BC) caused a significant reduction in the numbers of *Cx. quinquefasciatus* mosquitoes blood feeding (p=0.032; Table 8.1). Although the level of blood feeding inhibition was similar for the permethrin (CP) and *B. bassiana* (BC) treatments, the combined *B. bassiana* and permethrin (BP) treatment showed no additive or synergistic effects of these two individual treatments (Table 8.1). The logistic regression analysis found no significant effect of the day, indicating that the levels of blood feeding did not significantly vary during the trial.

Culex quinquefasciatus mortality seven days after being collected from the huts was fairly similar for all six treatments (Table 8.1). Even when taking into account the variation between the replicates caused by doing the trial over a relatively long period of time, there was no significant impact of the *M. anisopliae* (HR=1.03, p=0.85), *B. bassiana* (HR=1.12, p=0.45) or permethrin ITN (HR=1.02, p=0.87) treatments on the mortality of mosquitoes when compared to the respective control treatments. Furthermore, there were no significant interactions between the fungal and insecticide treatments.

There was also no significant difference between the numbers of mosquitoes found in the huts for the six treatment combinations (F=0.94, df=5,200, p=0.46) indicating no significant deterrence of any of the treatments. The six treatment combinations also all had similar levels of induced exophiliy (F=1.19, df=5,200, p=0.32), and immediate mortality (the numbers of mosquitoes that were collected dead from the huts) (F=0.35, df=5,200, p=0.88).

8.4.4 Fungal viability in the field

During the 20 day exposure of the treated pieces of netting to ambient field

conditions in Benin, the temperature range in the storage area where the nets were held was 24.9-38.6°C with humidity ranging from 70–>95%RH; daily means (±SE) were 30.0°C (±0.54) and 86%RH (±1.21).

The mean (±SE) initial viabilities of *B. bassiana* and *M. anisopliae* in their ShellSol T suspensions were 64% (±4.70; 2381 spores counted) and 8.5% (±2.62; 2339 spores counted), respectively. There was a steady and marked decrease in viability of the fungal conidia on the pieces of netting held under field conditions over time for both *B. bassiana* (Figure 8.4) (adjusted r^2=0.67, p=0.015) and *M. anisopliae* (Figure 8.4) (adjusted r^2=0.66, p=0.016) treated netting. After 16 days exposure to field conditions there were no viable conidia on the *M. anisopliae* treated netting, although this may be because the starting viability of the *M. anisopliae* used was just 8.5%, much lower than the viability of *B. bassiana* which was 64%.

8.4.5 Cone bioassays with fungus-treated netting

The mean (±SE) temperature and humidity during the bioassay exposure periods were 29.2°C (±0.44) and 90.6%RH (±1.52), with ranges of 27.2-32.1°C and 78->95%RH respectively.

Both species of fungi tested were pathogenic to the *An. gambiae s.s.* VKPER strain mosquitoes (Figure 8.5). Significantly increased mortality for the *B. bassiana*-exposed mosquitoes (when compared to the control mosquitoes), was seen when the treatment on the nets was 1, 3 and 5 days old (Table 8.2). For *M. anisopliae*-exposed mosquitoes, mortality was only significantly higher than the control when the treated netting had been exposed to the field conditions for 1 and 3 days; the *M. anisopliae*-treated netting left in the ambient field conditions for 5 days was unable to kill significantly more mosquitoes than the control netting (Table 8.2), again possibly due to the poorer quality of this fungal species production batch as shown by the lower starting viability.

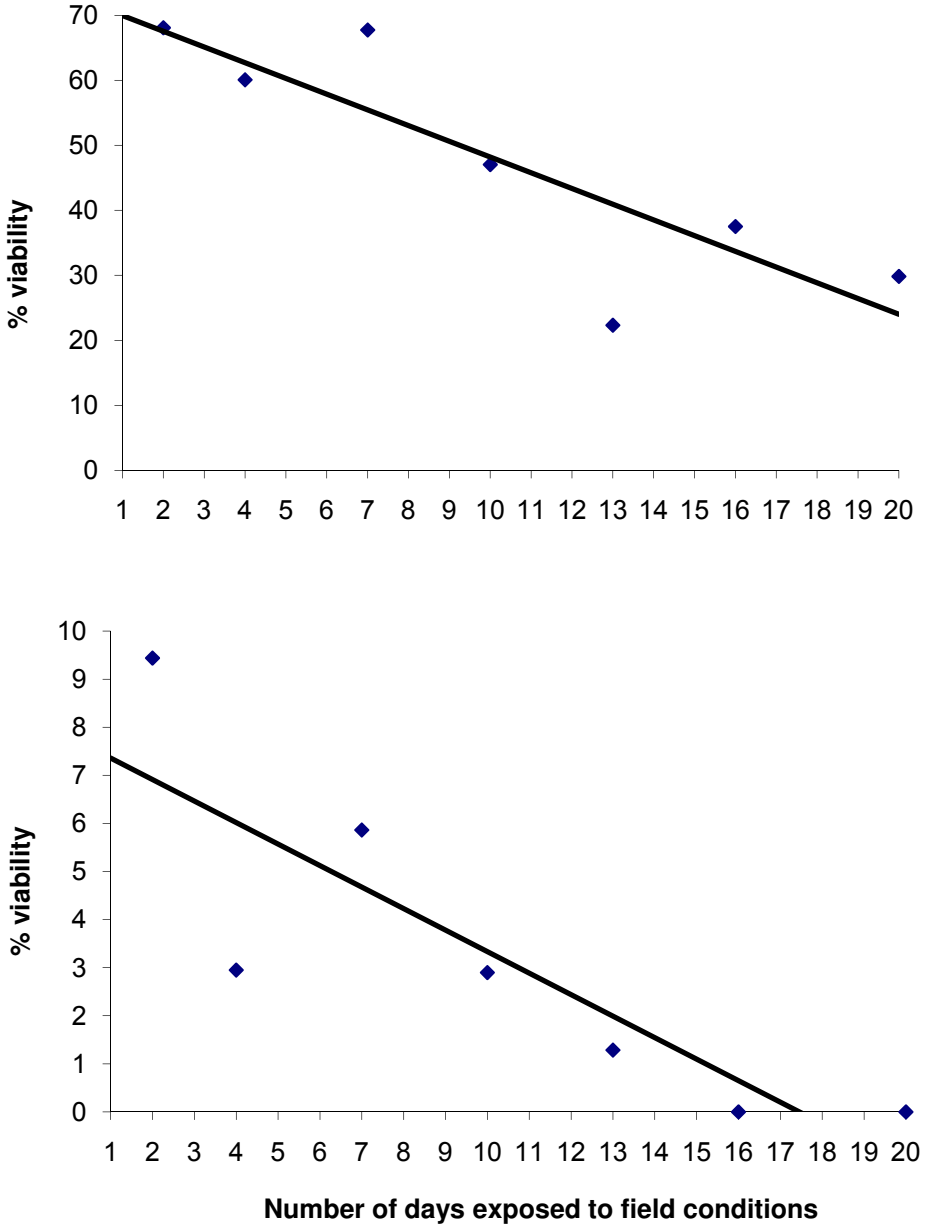

Figure 8.4. Percentage viability of spores of *Beauveria bassiana* (top) and *Metarhizium anisopliae* (bottom) on polyester netting exposed to field conditions for differing periods of time; lines represent the linear regression lines

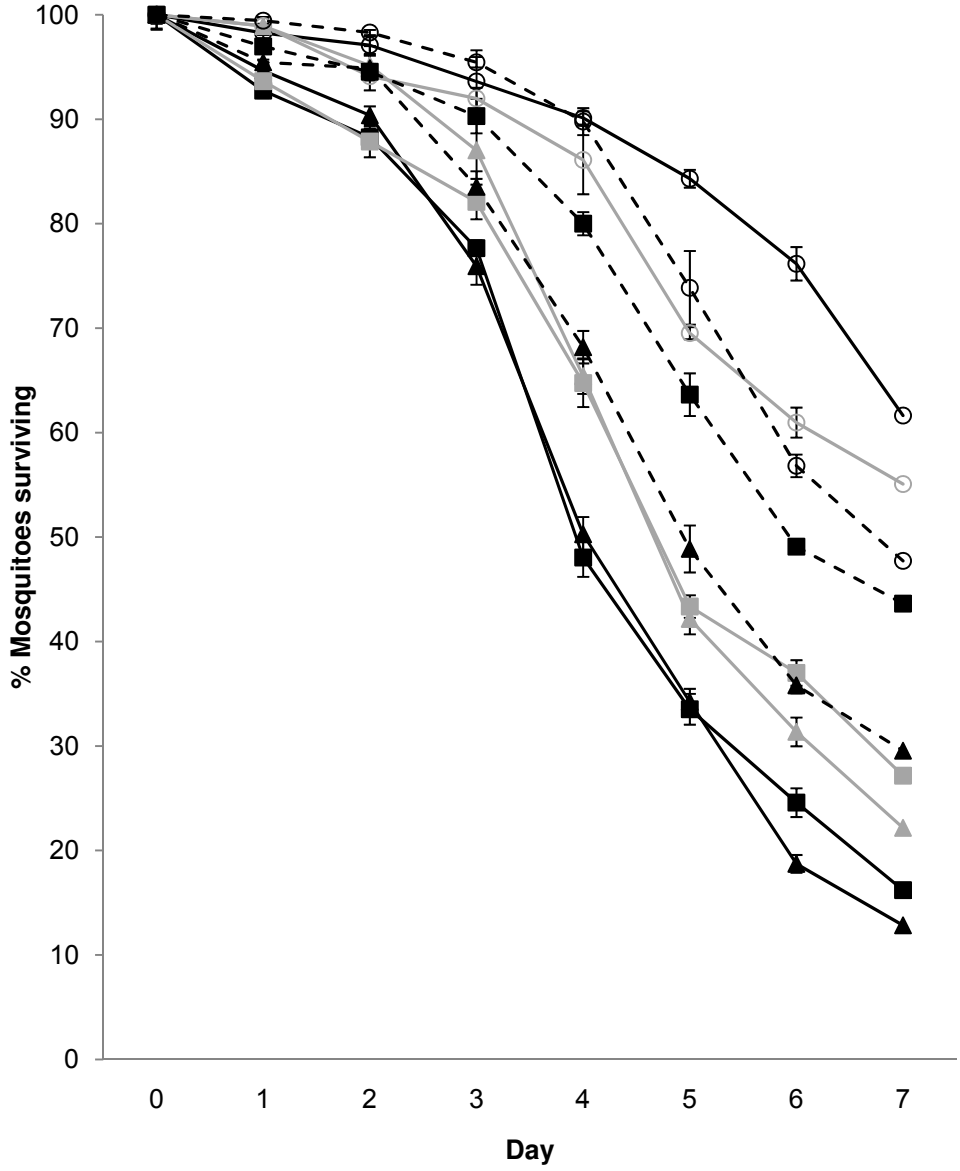

Figure 8.5. Mean (±SE) survival of *An. gambiae s.s.* VKPER mosquitoes exposed in WHO cone bioassays to fungus-treated netting exposed to field conditions for 1 (solid black), 3 (grey) and 5 (dashed black) days before testing. Control (open circles) mortality is compared to *M. anisopliae* (closed squares) and *B. bassiana* (closed triangles) induced mortality

Table 8.2. Survival analysis of *An. gambiae s.s.* VKPER mosquitoes exposed to two species of fungi in cone bioassays; data show Cox regression Hazard Ratio (HR) outcomes (95% CI), statistical p-values are relative to the relevant control

Fungus	**Time since fungal application (days)**	**HR (95% CI)**	**p**
M. anisopliae	1	2.41 (1.86, 3.12)	<0.0001
	3	2.14 (1.63, 2.82)	<0.0001
	5	1.20 (0.90, 1.60)	=0.206
B. bassiana	1	2.50 (1.94, 3.23)	<0.0001
	3	2.24 (1.71, 2.93)	<0.0001
	5	1.73 (1.32, 2.25)	<0.0001

Despite being significantly different from the control for all the time points, the virulence of the *B. bassiana*-treated net held in field conditions significantly reduced with increased time in the field; mosquito mortality caused by the one day old fungal treatment on the net was significantly higher than the mortality caused by the 3 day old (HR=0.75, p=0.014) and 5 day old fungal treatments (HR=0.673, p<0.001). Similarly, for *M. anisopliae* the mosquito mortality seen from the Day 1 net was significantly higher than that seen for the Day 3 (HR=0.75, p=0.017) and Day 5 nets (HR=0.50; p<0.0001). These results indicate a drop off of effectiveness with increasing time the fungal conidia spend exposed to ambient field conditions.

8.5 Discussion

Our study was the first to examine the effect of entomopathogenic fungi on wild

mosquito blood feeding in the field. In particular, the current study investigated more or less instantaneous impacts on feeding within a single feeding night (i.e. within a few hours of fungal exposure). The results show that *B. bassiana* treatments significantly reduced blood feeding, with *B. bassiana* alone able to inhibit 37% of blood feeding relative to the control. Permethrin was able to inhibit 43% of blood feeding, much higher than a previous study in the same study village where another pyrethroid, alphacypermethrin, reduced *Cx. quinquefasciatus* blood feeding by 27% (Irish et al. 2008). Given the results, it is unknown why no additive effect was seen in the blood feeding inhibitions when the *B. bassiana* (BC) and permethrin (CP) treatments were combined into the *B. bassiana* and permethrin (BP) treatment. Preventing blood feeding is important in terms of disease control and the finding that entomopathogenic fungi can prevent blood feeding in wild mosquitoes so soon after they acquired a fungal infection is both unexpected and important, but further research specifically targeting malaria vectors is required to substantiate this. Because blood feeding was significantly affected so soon after acquiring a fungal infection it is suggested that future application techniques for fungi in the field should target host seeking mosquitoes. If the fungi are deployed as post-feeding resting targets (Farenhorst et al. 2008), then one of the main ways in which entomopathogenic fungi can help reduce disease transmission would be missed.

Both of the fungal species used in our study have previously shown a propensity to reduce mosquito blood feeding under laboratory conditions. *Anopheles gambiae s.s.* exposed to oil-formulated *M. anisopliae* for 24 hours showed a reduced propensity to take subsequent blood meals when compared to uninfected mosquitoes; the proportion of mosquitoes taking a blood meal after fungal infection was reduced by 51% when compared to the control group (Scholte et al. 2006). This response may be linked to the down-regulation of genes controlling digestion in *An. gambiae* inoculated with *B. bassiana* (Aguilar et al. 2005) indicating that digestion and nutrient acquisition is not a priority for mosquitoes immediately after fungal infection. A different study using *B. bassiana*-infected *Anopheles stephensi*

Liston demonstrated that between days 8 and 14 post infection the fungus interfered with the ability of the mosquito to take a blood meal (Blanford et al. 2005). Although these earlier studies looked at feeding over several days following infection, they found a similar level of blood feeding reduction as found in our study. Relatively rapid changes in feeding behaviour after infection with *M. anisopliae* or *B. bassiana* have also been reported in many other insect types (Roy et al. 2006).

The mechanism behind the very rapid blood feeding inhibition observed in the current study is unknown but may be due to physiological and/or behavioural reasons. A mosquito may enter the huts several hours before blood feeding which would allow the fungus time to start germination and cuticle penetration. As far as we are aware no data have been published on the germination and penetration times on mosquito cuticles. However, in infected termites *M. anisopliae* germination occurred at 6 hours and penetration at 12 hours after infection; *B. bassiana* germination occurred between 6 and 12 hours post infection, with penetration between 12 and 24 hours post infection (Neves and Alves 2004). In this study, mosquitoes had to pass directly through the fungus-treated netting so some conidia could have got into the mosquito spiracles or at the base of the setae. This may decrease the fungal penetration time because the cuticle is thinner in these places (Neves and Alves 2004). Even during pre-penetration growth of the conidia the wax layer of the insect cuticle is degraded (Jarrold et al. 2007) and insects use both cellular and humoral immune responses against fungal infections starting as early as cuticle degradation (Gillespie et al. 2000). Therefore, if the germination and pre-penetration times on mosquito cuticles is similar to termites, then it is feasible that the immune system could have been activated during the short time the mosquitoes and sleepers were in the huts. Alternatively, the mosquito antennae may have become covered in conidia, interfering with their ability to detect the human host. In addition, termites have been shown to groom after fungal infection which successfully removes conidia (Yanagawa et al. 2009). This may also have taken place with our wild mosquitoes and could have interfered with their

host seeking.

The concept of an evolution-proof malaria control tool is to use a tool to kill mosquitoes after reproduction has taken place, but before the mosquito becomes infectious with the malaria parasites, allowing “susceptible” genes to perpetuate in the gene pool (Read et al. 2009). It is thought that entomopathogenic fungi could be such a tool. However, for this to occur, mosquitoes have to keep feeding through the duration of the fungal infection. Our results indicate that at least for a proportion of wild *Cx. quinquefasciatus* this would not occur, and laboratory work has shown similar effects in malaria vectors (Blanford et al. 2005, Scholte et al. 2006). Nevertheless, in many areas *Culex* mosquitoes are often more numerous than *Anopheles* and as such personal protection methods such as ITNs are often bought to prevent the nuisance biting as much as for any other reason. Failure to control these nuisance mosquitoes can reduce the uptake of ITNs for malaria control (Chandre et al. 1998, Kulkarni et al. 2007). Therefore tools that can reduce the biting of insecticide-resistant *Culex* mosquitoes are also required.

Despite the encouraging finding that *B. bassiana* can prevent blood feeding, no significant mortality was found in wild caught *Cx. quinquefasciatus* mosquitoes collected from the huts. Although previous findings have found that *Cx. quinquefasciatus* is susceptible to *M. anisopliae* (Scholte et al. 2003b, Scholte et al. 2005) it is important to note that *B. bassiana* has not previously been tested against adult *Cx. quinquefasciatus* mosquitoes either in the laboratory or field. Even after discounting the *M. anisopliae* data due to the extremely low viability under the field conditions of our study, the *B. bassiana* viability was within the range that could be used in the field in the future, but did not significantly impact wild mosquito mortality. There are three main reasons why this may be the case.

Firstly, the experimental method may have been ineffective at providing a sufficiently lethal dose to the wild mosquitoes, even though it was able to elicit a significant behaviour modification. Possible reasons for not being able to sufficiently infect wild mosquitoes include certain conditions affecting the conidia on

the netting, and the short contact time of the mosquitoes. It is unlikely that permethrin affected the conidia because it has been found not to be inhibitory to the various developmental stages of *M. anisopliae* (Mohamed et al. 1987), and viable conidia of *Metarhizium flavoviridae* stored in oil and lambdacyhalothrin were found long after the conidia stored in just oil had died (Sanyang et al. 2000). After as little as one week under field conditions dry conidia were seen to be released from the window netting in the huts. This quick evaporation of ShellSol T and release of conidia has also been found in Tanzania (Matt Kirby, Pers. Comm.) and may lead to a lack of conidial protection from the field conditions, and a decrease in the effective concentration. Using other oil formulations (Scholte et al. 2005) or encapsulation techniques may lead to higher conidial protection. Laboratory studies have shown that conidial viability is directly affected by the polyester netting (Howard et al. 2010) (**Chapter 7**), but even though the treated window netting proved effective at infecting mosquitoes in the laboratory, the ambient field conditions further negatively affected the conidia on the netting, as was evidenced by our viability and cone bioassay results. Temperature and humidity can adversely affect fungal conidia (Rangel et al. 2005, Lekimme et al. 2008, Darbro and Thomas 2009), however, the climatic conditions were similar for the experimental hut and bioassay nettings, so similar adverse effects would be expected. Nevertheless, fungal spores used in the bioassays were able to infect mosquitoes causing significant mortality, but those applied in the huts could not.

Scholte et al. (2005) found much higher levels of mortality in their field study where the *An. gambiae s.l.* mosquitoes were found resting on fungus-impregnated cotton cloths (Scholte et al. 2005). The short contact time with the hut fungal netting, although not an issue for *An. gambiae s.s.* mosquitoes in the behaviour experiments in the laboratory, could have caused problems because there appears to be a threshold number of conidia per unit surface area required for successful mosquito infection (Scholte et al. 2003b). This may be related to the up-regulated mosquito immune system being able to clear low-level fungal infections (Scholte et al. 2004a, Aguilar et al. 2005). If the proportion of viable conidia was decreased by

the polyester netting/field conditions then the wild *Culex* may not have been receiving enough viable conidia to initiate a successfully lethal fungal infection. We chose to target hut-entering mosquitoes as it was thought to be the most efficient use of the fungal conidia, and our laboratory studies confirmed that mosquitoes would pass through the netting, in the process picking up a fungal infection. Other proposed application methods in the field include cotton resting targets (Scholte et al. 2005), clay pots (Farenhorst et al. 2008), and odour baited stations (Lwetoijera et al. 2010), all of which will ensure longer contact times but would target resting mosquitoes post-feeding, and so may not affect blood feeding in the same way as the method used in this study.

The second reason for the lack of fungus-induced mortality could be that even if a successful fungal infection was received by the entering mosquitoes, then it is possible that the mosquitoes died of natural causes before any significant toxic effects of fungal infections could be seen because control survival was poor and this may have masked any effects of the fungus. After holding the mosquitoes for 7 days the control mortality was 54% and was not significantly different from the fungus and/or permethrin-exposed mosquitoes. The natural mortality could be quite high because the mosquitoes entering the huts were of an unknown age range, and insecticide resistance in *Culex* mosquitoes is known to be associated with fitness costs (Berticat et al. 2008) that can lead to reduced survival rates (Wang et al. 1998).

Finally, the third possible reason for the lack of fungal-induced mortality is that the wild multi-insecticide-resistant *Cx. quinquefasciatus* mosquitoes in Benin may just not have been susceptible to fungal infection. As mentioned, *Cx. quinquefasciatus* adults have not been previously shown to be susceptible to *B. bassiana* either in the laboratory or field. A previous laboratory study comparing *An. gambiae s.s.* and *Cx. quinquefasciatus* found very few differences in susceptibility to *M. anisopliae* infection, with both male and female *Cx. quinquefasciatus* having significantly reduced life spans after continuous exposure to both dry and oil-formulated conidia

(Scholte et al. 2003b). However, Scholte (2004) speculates that wild Tanzanian insecticide-susceptible *Cx. quinquefasciatus* in the field had higher immunocompetence towards *M. anisopliae* infection than wild *An. gambiae s.l.* because the infection rates were 10% and 33% respectively. Wild *Culex* may be less susceptible to fungal infection due to interactions of their micro-flora (Indiragandhi et al. 2007), or because their insecticide resistance mechanisms protected them (McCarroll et al. 2000).

Micro-flora interactions can protect insects from infection because the presence of mid-gut flora in *An. gambiae* has been shown to reduce malaria parasite infections by activating the immune system (Dong et al. 2009), and *Pseudomonas* bacteria found in insecticide-resistant diamond-back moths showed antagonistic activity against *M. anisopliae* and *B. bassiana* (Indiragandhi et al. 2007). The wild *Culex* mosquitoes in this study are likely to have gut flora that could possibly have affected fungal penetration and growth in a similar fashion. In addition, insecticide-resistant *Cx. quinquefasciatus* in Sri Lanka were shown to adversely affect the development of the filarial worm *Wuchereria bancrofti,* thought to be due to elevated esterase activity (McCarroll et al. 2000). Serebrov et al. (2006) found that infection of greater wax moth caterpillars with *M. anisopliae* caused elevated levels of esterases and GST, presumably as part of the immune response. If elevated esterase and GST levels are also an important immune response to fungal infection in *Cx. quinquefasciatus,* this would explain the low susceptibility of the wild mosquitoes in this study; in effect their immune system is already activated because they naturally have higher levels of these enzymes (Corbel et al. 2007).

Whilst the fungi were unable to cause significant mortality in wild caught *Cx. quinquefasciatus* mosquitoes collected from the huts, significant mortality was found in *An. gambiae s.s.* mosquitoes infected in WHO cone bioassays carried out under field conditions. A laboratory study has shown that even though fungal viability significantly decreases when placed onto white polyester netting, and again a week after application, the effectiveness of the fungal treatments at killing

mosquitoes was not significantly affected (Howard et al. 2010) (**Chapter 7**). Ultimately for mosquito control what is important is not necessarily viability, but whether the fungi can still infect and kill mosquitoes in the field. The cone bioassay results show that these fungi were able to cause significant mortality to an insecticide-resistant strain of *An. gambiae s.s.* after the fungi had been held under field conditions. Even the *M. anisopliae* was able to cause significant mortality when the netting had been held for 3 days, a time when the estimated viability would have been 6%. This raises questions as to whether the exposure time and concentration of the conidia was high enough to cause infection despite so few being viable, or is it linked to the agar/fungicide method underreporting the viability?

Fungal viability is a product of many variables including the production methods, the formulation used, the substrate treated and the climatic conditions, and viability is measured by plating conidia onto agar containing a fungicide. Whilst any of these variables can affect viability, our results showed that fungal viability decreased relatively rapidly when treated pieces of netting were left in ambient field conditions. Scholte et al. (2005) also found that the viability of *M. anisopliae* treated cotton sheets decreased from 96% in suspension to 63% three weeks after application in Tanzania. In addition, laboratory studies have also found relatively rapid losses in fungal viability after exposure to heat and humidity (Rangel et al. 2005, Lekimme et al. 2008, Darbro and Thomas 2009, Howard et al. 2010) (**Chapter 7**).

Not only did the viability on the netting decrease with increased time spent in the field, but the effectiveness of the fungal treatments to infect and kill malaria vectors also decreased with increasing exposure to ambient field conditions. More work needs to be carried out before the operational use of these fungi can become a reality. Questions still remain about the best application method, dose and the persistence of the fungal conidia under field conditions. Whilst our set up of polyester netting and ShellSol T proved effective under laboratory conditions

(Howard et al. 2010) (**Chapter 7**), it was evident that it was not an effective application method for use in tropical sub-Saharan African conditions. The major challenge in the use of entomopathogenic fungi for mosquito control is to translate the many successful and encouraging laboratory trials (Blanford et al. 2005, Scholte et al. 2006, Farenhorst et al. 2009, Howard et al. 2010) (**Chapter 7**) into field successes and develop an effective and sustainable field delivery system. Future research on fungal production methods, and testing new formulation and substrate combinations, should be carried out with a view to optimising these for eventual use in the field.

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Chapter 9

Summarizing discussion

9.1 Introduction

Malaria control is lagging behind expectations. African children are still dying of malaria every day and African communities need tools to tackle malaria now. Rural communities are the most at risk of malaria (Kirby et al. 2008, Kelly-Hope and McKenzie 2009), and have the least access to malaria control tools (Matovu et al. 2009). Therefore, the research described in this thesis focussed on addressing mosquito control from a rural perspective. Using mosquito control tools in a way that requires almost no technical equipment or knowledge will open them up to the rural communities that are best placed to deploy them. Employing these tools in this way may not be the most efficient way to use them, but at least the technology required is available where the tools are most needed. Many African communities would probably rather use a less efficient tool now than wait years for a more efficient tool to become available to them, because mosquitoes do not need to be controlled completely, just to a point where disease transmission can no longer or rarely occurs. As discussed in **Chapter 2,** there are many tools available to African communities for mosquito control. The research objective of this thesis was to examine the feasibility and effectiveness of the use of three natural products (flora, fish and fungi) for malaria vector control.

This thesis has answered many questions concerning the low-tech deployment of natural products and their potential use against insecticide-resistant mosquitoes. The use of these natural products for mosquito control is at varying stages of development; neem and fish should be operational and fungi could become operational in the future once certain issues are addressed. The chapters in this thesis describe many different experimental techniques that were used to examine different facets of these natural products. Laboratory and field studies have been used to examine the effect these products have on the mortality of larval (**Chapters 3 & 6**) and adult (**Chapters 7 & 8**) mosquitoes, and behavioural modifications have also been examined in the laboratory (**Chapter 4**) and field (**Chapter 8**). In addition, a field census was used to determine the current state of fish farming in

western Kenya (**Chapter 5**). From this wide range of experimental techniques, many promising results were obtained.

This chapter briefly explains why each of the natural products was chosen, what the main results of the thesis chapters were and puts the results in the context of malaria control. How the results compare to previous work has been discussed in detail in the preceding individual chapters. So as not to repeat the discussions made in the previous chapters, this final chapter focuses on giving recommendations for future work and drawing conclusions from the work undertaken. Finally, how the three F's (flora, fish and fungi) can be brought together in IVM programmes is discussed.

9.2 Part I: Flora

For the flora part of the thesis, the neem tree was chosen. Neem was briefly introduced in **section 2.3.3**, and was chosen as a natural product to investigate in this thesis for several reasons. The first reason is that several studies have previously proved that neem can kill mosquito larvae (Nagpal et al. 1995, Awad and Shimaila 2003, Okumu et al. 2007), and field studies have shown that it is also effective in natural field conditions (Rao et al. 1992, Gianotti et al. 2008). Another reason is that it already grows in many areas in Africa and is well known; the Swahili name for neem is 'mwarubaini' because it is thought that it can be used to cure 40 diseases. Therefore communities are already sensitised to the usefulness of this tree. The focus of this thesis was on natural products that could one day be used by local communities to control mosquitoes. I therefore wanted to use neem, but in a way that could easily be deployed in resource-poor rural areas of sub-Saharan Africa. For this reason it was decided to use crude aqueous extracts, essentially to see what would happen when neem wood was just soaked in water. Aqueous extracts were also used because they have been found to be less toxic to mammals than other neem extracts (Boeke et al. 2004b). This is important

because in many African countries, bodies of water that are potential mosquito breeding sites are also important for domestic water usage (Mutuku et al. 2006a, Imbahale et al. 2010), and therefore humans will invariably come into contact with larval mosquito control tools.

An extensive study of the larvicidal properties of neem wood had not previously been undertaken. We therefore decided to expose all aquatic stages to crude aqueous neem extracts in a series of laboratory experiments. We found that neem was able to effectively control larvae of the most efficient malaria vector in Africa (Costantini et al. 1999), namely *Anopheles gambiae* Giles *s.s.*, even at a relatively small dose. However, the dose required to control pupae was much higher and it is unlikely that this high dose would be used operationally just to target mosquito pupae (Howard et al. 2009) (**Chapter 3**). The study showed that neem wood placed into water leaches out phytochemicals that are able to kill mosquito larvae even though azadirachtin, thought to be the most active of neem's liminoids (Schmutterer 1995), was not detected in the aqueous extracts. In addition, this thesis reports the effect the aqueous neem extract has on adult mosquito behaviour. Monitoring adult behaviour is important because previous work has indicated that neem can repel female mosquitoes from ovipositing (Dhar et al. 1996). Our results showed that at a lethal dose for larvae, significantly more mosquitoes laid eggs when compared to control water-exposed mosquitoes. In addition, no significant repellent effects were seen even at doses 10x and 100x higher than the lethal dose for larvae (Howard et al. Under Review) (**Chapter 4**). Thus, mosquitoes were not prevented from exposing their progeny to the neem-treated water at any of the doses tested.

The results in this thesis can be seen as good news in the context of malaria control. The larval mortality (Howard et al. 2009) (**Chapter 3**) data proves that even wood of the neem tree (the part of the tree thought to contain the lowest concentration of active liminoids (Schmutterer 1995)) is able to control mosquito larvae at relatively low doses. Furthermore, this was achieved by simply placing the

wood into water, a method that requires almost no infrastructure and could easily be used in rural Africa. All doses used in the oviposition study (Howard et al. Under Review) (**Chapter 4**) allowed mosquitoes to still expose their progeny to the neem control tool. If this simple application of this control tool is to be used by rural communities, then the dose may not always be controlled. This could lead to overtly high doses being used, but our evidence suggests that even these very high doses will not adversely affect mosquito oviposition behaviour.

Neem has been tested extensively in the laboratory for the control of malaria vector mosquitoes (Ziba 1995, Mulla and Su 1999, Nathan et al. 2005, Howard et al. 2009) (**Chapter 3**), and field trials have been carried out using certain extracts (Nagpal et al. 1995, Awad and Shimaila 2003) and raw neem product (Gianotti et al. 2008). However, these studies only looked at entomological outcomes; the effect neem has on malaria transmission has not been tested yet. Field tests should be carried out to evaluate how the deployment of neem for mosquito control can directly impact malaria transmission. After this has happened, for the use of neem in general, there is no reason why the extracts already tested in field trials (Awad and Shimaila 2003, Gianotti et al. 2008) cannot be incorporated into integrated vector management (IVM) trials. If this happens, and it proves successful, the next stage would be to educate communities about the role that their popular shade tree could play in malaria control. Neem tree plantations could be planted in areas that are most at need of mosquito control, and this reforestation could help with issues like soil erosion. For the crude aqueous extracts of neem wood used in this thesis (Howard et al. 2009, Howard et al. Under Review) (**Chapters 3 & 4**), small scale field trials are required to see whether the extracts are able to effectively control mosquito larvae in the field; adult oviposition behaviour should also be monitored to see if the behaviour seen in the laboratory (Howard et al. Under Review) (**Chapter 4**) is replicated in the field because it may be that the oviposition response to neem is different in natural water bodies that produce a range of volatile signals. Although previous work has found that neem-based pesticides do not significantly affect non-target organisms (Kreutzweiser

1997), it is still important to monitor non-target organisms, such as invertebrate mosquito predators, to verify that the use of neem does not adversely affect the ecosystem.

In conclusion, even a simple aqueous extract of neem was able to control mosquito larvae at a relatively low dose (**Chapter 3**). That this ubiquitous tree is effective with such a simple application method is a promising result that paves the way for simple, cheap and potentially sustainable mosquito control that can be readily applied in the areas where it is most required. Furthermore, it was found that at the dose required for larval control, significantly more mosquitoes laid eggs when compared to the control. In addition, at higher doses mosquitoes were not significantly repelled from laying their eggs in the neem treated water (**Chapter 4**).

9.3 Part II: Fish

Fish were chosen as one of the natural products to be investigated in this thesis because fish farming is widespread in many African countries (FAO Inland Water Resources and Aquaculture Service 2003) and, as explained in **section 2.3.2**, many native fish are known to be larvivorous (el Safi et al. 1985, Louis and Albert 1988). In addition, fish are in situ in many malaria-prone areas, and this method of malaria vector control is already potentially at the operational stage.

For these reasons we conducted a fishpond census with the Kenyan Fisheries Department to examine the current status of fish farming in an area of western Kenya (Howard and Omlin 2008) (**Chapter 5**) to examine the effect that fish farming has on mosquito distribution and abundance. We found that fish farming was widespread and, as well as being a source of food and income, fish farming was able to control mosquito numbers in the field. Essentially fish farmers are already undertaking a form of mosquito control without knowing it. However, because they didn't realise the links between fish farming and malaria

transmission, they were prone to leaving ponds that were not stocked with fish and this leads to higher malaria vector abundance (Howard and Omlin 2008) (**Chapter 5**). Despite this, our results show that once the fish farmers were educated as to the risk these abandoned ponds posed, the demand for fish to restock them increased, highlighting the communities' willingness to participate in malaria vector control (Howard and Omlin 2008) (**Chapter 5**).

Furthermore, the census found that levels of abandonment were higher where incentives had been offered to start fish farming but then withdrawn. Clearly, incentives are not the way forward for sustainable mosquito control. A better method is education. Given the current status of fish farming, and the ability of *Oreochromis niloticus* L. (Perciformes: Cichlidae) to control mosquitoes (Howard et al. 2007) (**Chapter 6**), successful mosquito control could readily be implemented. However, if fish are to be actively used for malaria control, then the communities charged with maintaining the fishponds need to be sufficiently educated as to the role abandoned ponds have in mosquito proliferation. This education should address a range of issues as diverse as larval mosquito identification and ecology, and proper fish husbandry. Informal education of rural farmers via the Farmer Field School (**section 2.6**) has been a successful way to educate farmers about malaria transmission and the principles of IVM (van den Berg and Knols 2006, van den Berg et al. 2007) and I feel that a similar approach directed towards fish farmers would be of great benefit.

This thesis demonstrates that the most popular fish type farmed in western Kenya, namely tilapiine fish, are able to control mosquitoes. This was found by both passive (Howard and Omlin 2008) (**Chapter 5**) and active (Howard et al. 2007) (**Chapter 6**) methods. Active deployment of this fish was able to reduce malaria vector numbers by >94% when compared to the control pond, and the suppression of mosquito breeding continued for a number of months. This demonstrates that this edible fish can effectively and sustainably control malaria vectors (Howard et al. 2007) (**Chapter 6**). In the context of malaria control, the results from **Chapters 5**

& 6 are promising. Knowledge of fish husbandry is widespread, and there was a desire to restock ponds once farmers found out that abandoned ponds provided an ideal habitat for malaria vectors. In addition, communities are already familiar with *O. niloticus* because it is a popular food fish. Therefore, the deployment of this mosquito control tool should not have any issues with community acceptance. Furthermore, there should be a ready market for mature fish, which would allow this form of mosquito control to be driven by financial as well as entomological/health reasons. Crucially, this could lead to increased sustainability because malaria control tools that are linked to socioeconomic growth are more likely to be sustainable (Rajagopalan and Panicker 1985). It is for this economic reason that the deployment of *O. niloticus* is more appropriate for sustainable mosquito control than other larvivorous fish like the "mosquito-fish" *Gambusia affinis* Baird & Girard (Cyprinodontiformes: Poeciliidae). In addition, *O. niloticus* is a native African fish, and therefore should not be as destructive to African ecosystems as the non-native *G. affinis.*

The effective use of larvivorous fish for mosquito control has been demonstrated in field trials in several countries using many different types of fish (Wu et al. 1991, Fletcher et al. 1992, Ghosh and Dash 2007, Howard et al. 2007) (**Chapter 6**). The next step is for this mosquito control tool to be directly integrated into IVM trials and operational strategies. Whilst this has already occurred in India to great effect, where malaria was successfully controlled (Singh et al. 2006), for unknown reasons African countries have not yet embraced this readily accessible and potentially sustainable mosquito control tool for use as part of their malaria control arsenal. Experimental evidence has been collected showing that the operational use of fish for malaria control is successful and could be implemented now (Ghosh and Dash 2007). However, when choosing whether to implement the use of larvivorous fish for mosquito control, many different location-specific parameters need to be carefully considered (**Chapter 2**). One particularly important parameter is which native fish species are present, because the introduction of non-native species into ecosystems can have devastating results (World Health Organisation

2002). Also, as with all malaria control tools, the implementation of larvivorous fish should be accompanied by adequate participatory education to make it more acceptable for communities, and potentially more sustainable.

In conclusion, abandoned fish ponds lead to higher malaria vector abundance with the most dangerous vector, *An. gambiae s.l.*, being proportionally more abundant in abandoned ponds. Despite this, encouraging results for future malaria control were found. Knowledge of fish husbandry and the practice of fish farming is widespread (Howard and Omlin 2008) (**Chapter 5**), and the main fish farmed has been found to be highly larvivorous in a field trial (Howard et al. 2007) (**Chapter 6**). The only real remaining barrier for the use of larvivorous fish for mosquito control in Africa is adequate education. This education should be directed towards allowing farmers to realise the danger that abandoned fish ponds pose, and ways to find and treat the most productive mosquito breeding sites. For those that are unfamiliar with fish farming, fish husbandry techniques should be taught because fish will only thrive under certain optimum conditions (Trewavas 1983). The use of larvivorous fish can and should be readily integrated into IVM strategies.

9.4 Part III: Fungi

The potential of entomopathogenic fungi for mosquito control was introduced in **section 1.8.1,** and the development of this tool is at a less advanced stage than neem and fish. Nevertheless, this natural product was investigated in this thesis because it is currently the most promising natural product for the control of adult mosquitoes. Adult mosquitoes are important to target because of the relationship of certain parameters in the vectorial capacity equation (MacDonald 1957, Garrett-Jones 1964). Furthermore, widespread insecticide resistance is reducing the efficacy of current tools that target adult mosquitoes (N'Guessan et al. 2007). Because of the threat that insecticide-resistant mosquitoes pose, and the potential that fungi offer as a mosquito adulticide, we tested two species of

entomopathogenic fungi (*Metarhizium anisopliae* and *Beauveria bassiana*) against insecticide-resistant mosquitoes. In the laboratory studies, the insecticide-resistant mosquito strain was compared to an insecticide-susceptible strain (**Chapter 7**), and in the field wild insecticide-resistant mosquitoes were targeted (**Chapter 8**).

The laboratory work in this thesis was the first to use a fungal infection method subtle enough to explore individual strain effects between insecticide-resistant and insecticide-susceptible mosquitoes. The results showed that the insecticide-resistant strain *An. gambiae s.s.* VKPER was significantly more susceptible to entomopathogenic fungi than the insecticide-susceptible strain *An. gambiae s.s.* SKK (Howard et al. 2010) (**Chapter 7**). The tendency to kill insecticide-resistant mosquitoes faster than insecticide-susceptible ones should be of benefit when tackling insecticide-resistance in the field, because fungal infection will quickly remove insecticide-resistance genes from the population while leaving the insecticide-susceptible mosquitoes to breed, which is important for keeping the fungus “evolution-proof” (Read et al. 2009).

As well as exploring the effects of fungal infection in insecticide-resistant mosquitoes, this thesis focussed on assessing a potential application method that could be used for fungal conidial delivery in the field. This proposed application method was conidia suspended in ShellSol T and applied to white polyester netting. Whilst we found that this application method did not significantly alter the ability of conidia to infect and kill malaria vectors in the laboratory (**Chapter 7**), problems arose under field conditions (**Chapter 8**). In the laboratory, ShellSol T has previously been shown to be an effective delivery tool for fungal conidia (Farenhorst and Knols 2010), and this was also found in our laboratory study (**Chapter 7**). However, under field conditions the ShellSol T appeared to evaporate quickly and the fungal conidia were released from the netting. Furthermore, although the polyester netting was shown to adversely affect fungal viability in the laboratory this did not alter the effectiveness against mosquitoes (Howard et al. 2010) (**Chapter 7**). However, in the field the ambient climatic conditions also

adversely affected fungal viability to a point where the effectiveness against mosquitoes was compromised (**Chapter 8**). Whilst these studies have shown that the use of ShellSol T and white polyester netting is not an appropriate delivery method of entomopathogenic fungi in the field, the more important message is that laboratory work is no substitute for field work. This is apparently true even when doing laboratory tests under conditions designed to mimic field conditions, as in this thesis.

The field component of the fungal part of this thesis (**Chapter 8**) was the first time entomopathogenic fungi have been used against wild insecticide-resistant mosquitoes. It was also the first time fungi have been used in West Africa specifically to target mosquitoes, and the first time adult mosquitoes have been targeted with *B. bassiana* in the field. In fact, this study was the first time *B. bassiana* has been used to infect *Culex quinquefasciatus* Say either in the laboratory or field. In addition, it was the first study anywhere in the field to investigate and measure how entomopathogenic fungi affect the blood feeding of wild mosquitoes. This study also reports the first use of entomopathogenic fungi in a WHO cone bioassay set up. Given our conclusions following the laboratory work - that polyester netting could be used to infect mosquitoes with fungal conidia - we used this application method in the field in a World Health Organisation (WHO) recommended phase II experimental hut study (World Health Organisation 2006). Because there were not enough malaria vectors present in the study area during the trial, analysis was carried out on the nuisance mosquito *Cx. quinquefasciatus.* No significant effect on mosquito mortality was found, but *B. bassiana* was able to significantly reduce the blood feeding of *Cx. quinquefasciatus* when compared to the blood feeding in the control hut (**Chapter 8**). The WHO cone bioassays, carried out under ambient field conditions, found that entomopathogenic fungi applied on polyester netting could be used to infect and kill insecticide-resistant malaria vectors under field conditions.

Given the present state of fungal research, it is only possible to talk about these

findings in the context of the potential for future malaria control, rather than actual ready deployment possibilities. Our (Howard et al. 2010) (**Chapter 7**) and previous laboratory findings (Farenhorst et al. 2009, Kikankie et al. 2010) have proven that fungi could be used to control insecticide-resistant malaria vectors, but, for unknown reasons, this was not found in the present study when exposing wild insecticide-resistant *Cx. quinquefasciatus* mosquitoes to fungal entomopathogens (**Chapter 8**). Our field results did show that fungi can be used to suppress mosquito blood feeding very soon after a fungal infection was acquired (**Chapter 8**). This reduction of *Culex* blood feeding could play a role in malaria control because *Culex* are often implicated in the success or failure of malaria control campaigns because they are often more numerous than *Anopheles,* and as such, personal protection methods such as ITNs are often bought to prevent the nuisance biting as much as for any other reason (Chandre et al. 1998, Kulkarni et al. 2007). Furthermore, although not referring to malaria vectors, this instantaneous reduction in blood feeding is a very interesting finding, and if it can also be found in malaria vectors then the use of entomopathogenic fungi in malaria control programmes will become even more interesting, and their development will become more pressing.

Many laboratory studies have been carried out using entomopathogenic fungi, and there is little doubt that fungal conidia can infect and kill malaria vectors under controlled laboratory conditions (Blanford et al. 2005, Farenhorst et al. 2009, Howard et al. 2010) (**Chapter 7**). However, only three field studies have been undertaken to date (Scholte et al. 2005, Lwetoijera et al. 2010) (**Chapter 8**), and none of these have directly monitored the effect of entomopathogenic fungi on malaria disease dynamics. The field studies so far undertaken have shown that whilst the fungal treatments have been effective at killing wild *Anopheles* mosquitoes (Scholte et al. 2005, Lwetoijera et al. 2010) and modifying the behaviour of wild *Culex* mosquitoes (**Chapter 8**), conidial viability decreases in a matter of just weeks (Scholte et al. 2005) (**Chapter 8**). Therefore, further field trials need to be undertaken specifically looking at maximising conidial viability under

field conditions, and developing an application method that can withstand field conditions and effectively deliver fungal conidia for a long period of time. Once these issues have been addressed, field trials directly examining the effect entomopathogenic fungi have on malaria transmission have to be carried out.

In conclusion, the results in this thesis show that insecticide-resistant *An. gambiae s.s.* mosquitoes are more susceptible to fungal infection than insecticide-susceptible mosquitoes (**Chapter 7**). This leads to interesting possibilities involving population dynamics and the conservation of insecticide susceptibility genes. While the delivery method of ShellSol T and polyester netting worked well in the laboratory, it was unable to adequately protect conidial viability in the field. Not enough malaria vectors were collected to allow the impact of fungi to be analysed in the field, but analysis of *Cx. quinquefasciatus* showed no fungal-induced mortality (**Chapter 8**). This may be due to an inadequate experimental method or high natural mortality. It is interesting to note that no previous study has exposed *Cx. quinquefasciatus* mosquitoes to *B. bassiana* and, therefore, they may just be innately resistant to this fungal species. The instantaneous effect on blood feeding (**Chapter 8**) is interesting and should be further examined using wild malaria vectors. In terms of further work, the priority must be on field studies and work that prolongs the efficacy of conidial viability under natural field conditions, and that can effectively assess a robust method of delivering the fungi under tropical conditions.

9.5 The 3 F's together in the context of IVM

Future work should be directed towards combining these and other methods in IVM trials with the view to measuring both entomological and malaria-case outcomes. How certain mosquito control tools can be combined in IVM programmes was discussed in **Chapter 2**. In this final chapter, more focus will be given to the combination of flora (specifically neem), fish and fungi.

The use of raw neem product to produce crude aqueous extracts in the field can be combined with many other types of mosquito control. It can be used with all forms of adult control, however, in terms of larval control more restrictions apply. Neem cannot be used in the same water bodies as larvivorous fish, because neem has been shown to adversely affect a range of fish species (Mondal et al. 2007, Winkaler et al. 2007) including the "mosquito fish" *G. affinis* (El-Shazly and El-Sharnoubi 2000, Awad 2003). One mosquito control tool that neem should work well with is environmental management. The number of possible larval breeding sites can be reduced through environmental management, but some communities need these mosquito-friendly sources of water (Mutuku et al. 2006a, Imbahale et al. 2010). Under these circumstances, neem wood could be placed in cotton sacks (to allow the phytochemicals to leach out) in the remaining water bodies. This would provide larval control in these areas but, more importantly, if these water bodies were to dry out and then re-flood, the neem would still be there and the phytochemicals could leach out into the newly deposited water. This form of control is not possible with a range of other larval control tools and is a major benefit of the raw neem product approach.

There are also some restrictions on the use of larvivorous fish. As mentioned above, they cannot be used in the same water body as neem. Furthermore, they cannot be successfully used in water bodies that are prone to dry out. Therefore, fish should be used for the larger and more permanent water bodies and neem can be used in the water bodies liable to drying and flooding. Also, as mentioned in **section 2.5,** fish are not compatible with the conservation of invertebrate mosquito predators (el Safi et al. 1985, Louca et al. 2009) or with the use of chemical insecticides (Walton 2007, Jayasundara and Pathiratne 2008). Despite these restrictions, larvivorous fish have been incorporated into successful IVM programmes, most notably in India where the use of larvivorous fish is a critical component of the Urban Malaria Scheme and the Enhanced Malaria Control Project (Chandra et al. 2008).

The deployment of entomopathogenic fungi for adult mosquito control, when operational, could be combined with almost all existing mosquito control tools. A recent model looking at the simultaneous application of entomopathogenic fungi and insecticide-treated bednets (ITN) predicts that in situations with low ITN coverage a synergistic effect of the fungal conidia and ITNs will be found; in situations of high malaria transmission intensity or insecticide resistance, and high ITN coverage, fungal applications are predicted to be very effective even at low fungal coverage (Hancock 2009). Furthermore, pyrethroid insecticides do not affect fungal conidia (Sanyang et al. 2000) and so entomopathogenic fungi could also be used in an IRS set-up. In theory, the use of fungi for adult mosquito control could also be combined with the use of neem and fish, because the latter two target the larvae, and fungi would be used to target adults. Tests need to be carried out combining fish and/or neem with fungi to see whether they will affect the use of entomopathogenic fungi for larval mosquito control (Bukhari et al. 2010).

9.6 And finally....

A wide range of natural products were used in this thesis because the future of malaria control does not lie with one “silver-bullet” method. Many different methods need to be used simultaneously in IVM programmes. In that regard, all of the natural products tested in this thesis can be incorporated into IVM programmes. Furthermore, the products tested can be used to decrease reliance on insecticides, and therefore potentially increase the sustainability of the IVM programmes. In addition, the use of natural products could allow successful mosquito control in areas where high levels of insecticide resistance have been reported. Crucially, two of the products tested in this thesis (flora and fish) have the potential to bring sources of income to the rural communities that are at most risk from malaria, and where IVM programmes will initially be focussed. These two products have proved effective in field trials and could be implemented now.

Control programmes incorporating natural products that lead to successful mosquito suppression, along with an increase in the socioeconomic status of the community, not only have the potential to be more sustainable than some top-down insecticide-based control programmes, but they can also lead to an increased sense of understanding, ownership and empowerment among the community. This is important because eventually African communities will be charged with monitoring and implementing mosquito control. This process will be made easier if the control tools used are already familiar to the communities and are readily available, such as neem trees and larvivorous fish. However, for these tools to be used effectively, rural communities must be taught the importance of mosquito control, and how to use these techniques cheaply and effectively.

The successful future of malaria control, and ultimately elimination, lies in engaging, empowering and entrusting rural communities with mosquito control in their environment using many methods in an IVM approach. For this to occur, cheap, readily accessible and effective mosquito control tools, such as those investigated in this thesis, need to be researched and developed. When the use of these tools becomes operational, the emphasis must be on bridging the knowledge gap between the scientific and rural communities that bear the brunt of the malaria burden. This is the real challenge of effective and sustainable malaria control. The message about effective mosquito control, backed up with scientific results, needs to be spread to the affected communities to fulfil the real purpose of scientific exploration. The status quo can only be changed when the communities are fully aware of the ways in which they themselves can help prevent mosquito breeding and malaria transmission, and when these communities are given the tools and knowledge for sustainable mosquito control and malaria prevention. Communities at risk of malaria need to be fully informed and educated about these things, because “education is the most powerful weapon which you can use to change the world” (Nelson Mandela).

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Samenvatting

Inleiding

Ondanks veel aandacht in de afgelopen 10 jaar van internationale organisaties voor malaria, sterven nog steeds grote aantallen Afrikanen, vooral kinderen, aan deze ziekte. Het is duidelijk dat malaria slechts succesvol kan worden beheerst met behulp van verschillende controle-instrumenten toegepast door middel van geïntegreerd vector management (IVM), en dat de Afrikaanse gemeenschappen veel meer rechtstreeks betrokken moeten worden bij muggen-bestrijding (**hoofdstuk 2**). Het gebruik van middelen om muggen te bestrijden op een manier die weinig technische apparatuur of kennis vereist zal deze beschikbaar maken voor de landelijke gemeenschappen op de plek waar deze het meest effectief zijn. Wijdverspreide insecticidenresistentie vermindert de effectiviteit van op insecticide gebaseerde instrumenten voor muggenbestrijding. Om deze redenen, worden biologische bestrijding en andere natuurlijke muggenbestrijdingsmethoden onderzocht door veel verschillende instellingen. Verschillende potentiële natuurlijke beheersinstrumenten zijn voorhanden in sub-Sahara Afrika. Als deze instrumenten effectief ingezet worden, dan kan dit een duurzame oplossing zijn, omdat gemeenschappen de biologische agentia zelf kunnen produceren, waardoor een bron van inkomsten voor landelijke gemeenschappen ontstaat. Dit zou vooral belangrijk zijn in gebieden waar de infrastructuur slecht is ontwikkeld, en herhaald gebruik van chemische beheersinstrumenten niet gemakkelijk kan worden toegepast. Dit proefschrift was bedoeld om de haalbaarheid en de effectiviteit van een verscheidenheid aan natuurlijke producten tegen zowel de larven als tegen de volwassen malaria muggen te testen met behulp van low-tech methoden in het laboratorium en door middel van veldproeven.

Deel I: Flora

Azadirachta indica A. Juss (Meliaceae) (de neem-boom) is gekozen vanwege de reeds bewezen mugdodende eigenschappen, en zijn beschikbaarheid in Afrika. We wilden de neem-boom gebruiken op een manier die gemakkelijk kan worden ingezet in plattelandsgebieden met weinig middelen. Laboratorium studies werden uitgevoerd om de larf en pop dodende eigenschappen van een waterig extract van ruw neemhout tegen de belangrijkste Afrikaanse malaria vector, *Anopheles gambiae* Giles *s.s.* (Diptera: Culicidae) te onderzoeken (**hoofdstuk 3**). De resultaten geven aan dat zelfs een relatief lage dosis van 0,15 gram gedroogd neemhout in 1 liter water in staat was de ontwikkeling van 90% van de muggenlarven tot volwassenen tegen te houden als muggenlarven werden blootgesteld tijdens hun eerste drie larvale stadia. Zelfs voor de vierde (laatste) instar larven was slechts 0,6 g / l nodig om de ontwikkeling van 90% van de muggenlarven tot volwassenen tegen te houden. Bovendien nam de ontwikkelingsduur van neem blootgestelde larven aanzienlijk toe in vergelijking met de controles. Poppen werden ook gedood door de waterige neem extracten en kregen neem geïnduceerde afwijkingen. De concentraties die nodig zijn om poppen te doden waren echter veel hoger dan voor de larven en zullen waarschijnlijk niet operationeel worden gebruikt. High-performance liquid chromatography (HPLC) analyse identificeerde verschillende polaire bestanddelen in de waterige neem extracten waaronder nimbin en salannin. Azadirachtine was echter niet in aanzienlijke hoeveelheden aanwezig. Het effect van dit extract op het ovipositiegedrag van een volwassen *An. gambiae s.s.* vrouw werd vervolgens bestudeerd (**hoofdstuk 4**). De ovipositie resultaten tonen aan dat bij gebruik van 0,1 g / l van het ruwe waterige neem-extract, aanzienlijk meer muggen hun eieren leggen vergeleken met de controle behandeling. Er was geen verschil tussen de controlegroep en de behandelingen met een 10- en een 100-voudige dosis voor wat betreft het aantal muggen dat eieren legde. Hieruit blijkt dat zelfs bij veel hogere doseringen dan nodig voor een succesvolle bestrijding van larven, de ovipositie niet nadelig wordt beïnvloed.

Deel II: Vis

Larf etende vissen zijn een bewezen methode om muggenaantallen te beheersen. Daarom werd een telling uitgevoerd om de huidige status van de visteelt in het westen van Kenia (**hoofdstuk 5**) te onderzoeken. Werkend met het Keniaanse Ministerie van Visserij merkten we dat, alhoewel het kweken van vis een favoriete activiteit is, 30% van de 261 gevonden vijvers geen vis bevatten. Deze "verlaten" vijvers hadden aanzienlijk meer *An. gambiae s.l.*, *Anopheles funestus* Giles en culicine muggen in vergelijking tot de vijvers die nog wel vis bevatten. *Anopheles. gambiae s.l.* was verhoudingsgewijs meer aanwezig in de verlaten vijvers vergeleken met de andere soorten muggen. Verrassend genoeg had vegetatie geen significante invloed op de aanwezigheid van muggen. Na onze studie, steeg de vraag naar vis voor de vernieuwing van verlaten vijvers met 67% ten opzichte van het voorgaande jaar. De overgrote meerderheid van de gekweekte vis in ons telgebied waren vissen van de tilapiine onderfamilie. Daarom, stelden we een kleinschalig veldonderzoek in naar de larf etende capaciteit van de vis *Oreochromis niloticus* L. (Perciformes: Cichlidae) (**hoofdstuk 6**). Door dagelijkse tellingen van de mugaantallen, zagen we dat direct na introductie van de vis, de dichtheid van muggen in de behandelde vijvers daalde in tegenstelling tot een toename in de controle vijver. Na 15 weken waren de aantallen *Anopheles* gedaald met > 94% in de vijvers met de vis. Zo werden muggenaantallen duurzaam beheerst tijdens de zes maanden durende studie. Geconcludeerd wordt dat deze vissensoort een effectieve en duurzame manier biedt om muggenaantallen in het westen van Kenia te beheersen. Bovendien, vormt deze vis een bron van broodnodige inkomsten en eiwitten aan Afrikaanse plattelandsgemeenschappen.

Deel III: Schimmels

Voor de controle van de volwassen mug met behulp van natuurlijke producten zijn

entomopathogene schimmels het meest veelbelovend. In dit proefschrift worden de entomopathogene schimmels *Beauveria bassiana* en *Metarhizium anisopliae* afzonderlijk in een minerale olie suspensie gebracht en toegepast op polyester gaas. Vervolgens werd een laboratorium experiment uitgevoerd om de gevoeligheid voor schimmels van insecticidengevoelige en insecticidenresistente stammen van *An. gambiae s.s.* te onderzoeken. Daarnaast werd de levensvatbaarheid van schimmelsporen getest op verschillende tijdstippen na de toepassing op polyester gaas (**hoofdstuk 7**). Hoewel beide mug stammen gevoelig waren voor beide soorten schimmels, was de pyrethroïde-resistente *An. gambiae s.s.* VKPER stam aanzienlijk gevoeliger dan de insecticide-gevoelige SKK stam, en stierf sneller. De levensvatbaarheid van schimmelsporen was significant lager voor beide soorten na toepassing op het polyester gaas, in vergelijking met de levensvatbaarheid in suspensie. Echter, het vermogen van de behandelde netten om muggen te infecteren en te doden was niet significant afgenomen tijdens de proefperiode van een week. Gezien de constatering dat met schimmel behandeld polyester gaas muggen kan infecteren en doden , werd een experimenteel veldonderzoek met hutten uitgevoerd in Benin, West-Afrika, teneinde het effect van de schimmelbehandeling op bloed eetgedrag en het voortbestaan van wilde insecticidenresistente muggen te onderzoeken. Benin werd gekozen vanwege de aanwezigheid van multi-insecticideresistente muggenpopulaties die een bedreiging vormen voor de effectiviteit van de huidige vector controle. We gebruikten een met schimmel behandeld net om muggen die in de hut kwamen te infecteren, in combinatie met of een onbehandelde of een met insecticide behandelde klamboe in elke hut om zo te onderzoeken hoe de entomopathogene schimmels werken met de huidige controle-instrumenten (**hoofdstuk 8**). Er werden alleen genoeg *Culex quinquefasciatus* Say (Diptera: Culicidae) muggen verzameld uit de hutten voor een nauwkeutige analyse. Onze studie is de eerste die het effect van entomopathogene schimmels op de bloedmaaltijd van wilde muggen bestudeerde. We vonden dat de *B. bassiana* behandelingen aanzienlijke en onmiddellijke vermindering van bloed voeden veroorzaakt. Er werd geen significant effect van de

schimmels op muggen mortaliteit gevonden. De spoorlevensvatbaarheid van *B. bassiana* en *M. anisopliae* bleek snel af te nemen onder veldomstandigheden.

Conclusies

Dit proefschrift beschrijft verschillende experimentele technieken om het potentieel van drie natuurlijke producten voor mugbeheersing te onderzoeken. Voor de flora, bleek dat zelfs een kleine hoeveelheid neemhout in het water mugpopulaties zou beheersen (**hoofdstuk 3**), en bij een hogere doses, werd het ovipositie gedrag niet nadelig beïnvloed (**hoofdstuk 4**). Neem bomen zijn verkrijgbaar in vele gebieden van Afrika, en uit veelbelovende veldproeven blijkt dat het gebruik van deze boomsoorten moet worden opgenomen in de bestrijding van malaria.

Dit proefschrift rapporteert dat de eetbare inheemse Afrikaanse vissen effectief kunnen zijn voor het beheersen van mugpopulaties (**hoofdstuk 6**), maar als de vijvers voor het kweken van vis zijn verlaten, dan kunnen grote aantallen van de meest effectieve malariavectoren hier broeden (**hoofdstuk 5**). Vissen zijn met succes gebruikt voor malaria vector beheersing in vele landen en dit kan worden toegepast op geschikte terreinen in Afrika, zolang dit gepaard gaat met een adequate voorlichting over de gevaren van verlaten vijvers.

We vonden dat insecticidenresistente muggen vatbaarder waren voor schimmelinfecties dan de insecticidengevoelige stam. Onder veldomstandigheden waren schimmels in staat om bloed voeden van muggen te voorkomen, maar veroorzaakten geen grote sterfte in gevangen wilde muggen. Hoewel entomopathogene schimmels een hoog niveau van sterfte veroorzaken in het laboratorium, (**hoofdstuk 7**), heeft het gebruik ervan onder veldomstandigheden nog een lange weg te gaan en is nog niet in de operationele fase. Hoewel de resultaten gevonden in dit proefschrift bemoedigend zijn voor het gebruik van schimmels in de Afrikaanse situaties (**hoofdstuk 8**), moet verder werk worden uitgevoerd om schimmelpersistentie onder veldomstandigheden te maximaliseren.

De huidige nadruk ligt op IVM voor de bestrijding van malaria (**hoofdstuk 2**), en de focus is gericht op de biologische beheersinstrumenten die kunnen helpen bij het beheren van insecticidenresistente populaties. Met dit in gedachten hebben de natuurlijke producten onderzocht in dit proefschrift bemoedigende resultaten opgeleverd die laten zien dat ze het potentieel hebben om te worden geïntegreerd in strategieën voor de bestrijding van malaria. Bovendien zijn de flora en vissen direct beschikbaar in de gebieden waar zij het meest nodig zijn, en kunnen vrijwel direct worden gebruikt om mugaantallen te helpen verminderen en daarmee de overdracht van malaria.

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I first become really interested in malaria during a Bob Sinden lecture at Imperial College. We then sat for seemingly hours in his office discussing different mosquito control techniques and capture methods. It was Bob who wrote my reference to the London School of Hygiene and Tropical Medicine (LSHTM), and it must have been a good one because I was awarded the Mansfield Aders Scholarship and Avebury Memorial Fund Award. So, a big thanks to Prof. Bob Sinden. Once at LSHTM I was helped by a great number of people. My tutor Mark Rowland was a great source of inspiration and cemented in my mind that I wanted to carry out mosquito field work. Chris Curtis was hugely knowledgeable, approachable and always had time for the students. I remember one time I had a strange mosquito question and for once, Chris didn't know the answer. The next think I knew, he had phoned Alan Clements (of the "Biology of Mosquitoes" textbook fame) so that I could ask him direct! Jo Lines was also a great help as were all the other scientists and backroom staff at LSHTM. I want to thank them all.

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Curriculum Vitae

On the 3rd December 1979, I, Annabel Frances Victoria Howard, was born in London, the UK. I grew up in Gloucestershire on a sheep farm, and as an 8 year old was fascinated with the wiggly creatures in the sheep troughs. Little did I know that I would devote my life to those wiggly things, because it was only later that I found out that they were mosquito larvae. I did my A levels at Rendcomb College and then went to Imperial College, London, to study Biology; I graduated in 2001. It was at Imperial that I developed an interest in malaria and mosquitoes because Bob Sinden was talking about using mosquitoes as "flying hypodermic needles". This captured my imagination and I there and then decided that I would focus my studies on mosquitoes. In this capacity, I did my BSc thesis in the laboratory of Andrea Crisanti, also at Imperial, and compared the biology of transgenic and wild type *Plasmodium berghei* in *Anopheles stephensi* mosquitoes. Prof Crisanti offered me a PhD placement, but I knew that I didn't want to do molecular genomics of transgenic malaria parasites, so I turned it down.

After leaving Imperial College I took a year out to travel the world and decide what it was I really wanted to do. It was during this time that both the parasite and mosquito genomes were published in *Nature* and *Science* respectively. Whilst trying to hunt down a copy of these journals in paper shops in New Zealand, I realised that my future did lie with malaria, and more specifically I wanted to focus on mosquitoes. Part of my reasoning for this was that the parasites could easily be studied in most laboratories, but to study mosquitoes you had to be out and about in the world and that appealed to me.

I applied to the London School of Hygiene and Tropical Medicine (LSHTM) for a place on an MSc course titled "The Biology and Control of Disease Vectors" (BCDV). I was lucky enough not just to be accepted, but also to be awarded the Mansfield Aders Scholarship and Avebury Memorial Fund Award. I studied at

LSHTM from 2003-2004 and met many great names in the field of Medical Entomology. Mark Rowland was my tutor and helped enormously, allowing me to work on one of his projects in Moshi, Tanzania for my MSc thesis investigating pyrethroid resistance in the wild *Anopheles arabiensis* mosquito population. I finished my MSc at LSHTM in 2004, graduating at the top of my class with a Distinction.

Whilst writing up my MSc thesis, I had been told about an organisation out in Kenya that did the kind of work I was interested in. I started working for the International Centre for Insect Physiology and Ecology (icipe) in 2005 and ended up staying with them for nearly 3 years. During this time I worked in Kisii and Kisumu in Kenya, working on the use of fish and neem to control mosquitoes. It was whilst at icipe that I started publishing my work. My first paper, published in 2007 in BMC Public Health (**Chapter 6**), was shortlisted for the Medicine Prize in the BioMed Central Research Awards in 2007. As well as the work in the **Chapters 3, 4, 5 & 6,** I was also involved in other field studies and was the senior entomologist in the Kisii/Kisumu field stations. For part of my time I was stationed at the KEMRI/CDC compound in Kisumu, and was able to work and rub shoulders with even more giants from the Medical Entomology field. I lived permanently in Kenya during this time, and developed an appreciation for the real issues rural African communities were facing.

My next career move in 2008 was to the Netherlands to work at Wageningen University with Bart Knols, Willem Takken and Marcel Dicke, amongst others. I used entomopathogenic fungi to infect insecticide-resistant mosquitoes (**Chapters 7 & 8**) and had the opportunity to carry out field work in Benin with Raphael N'Guessan. I also set about writing up this PhD thesis.

Having already worked in the UK, Tanzania, Kenya, the Netherlands and Benin, I am excited to find out where I will end up working next....

Correspondence: afv.howard@gmail.com

List of Publications

Howard A. F. V., G. Zhou, and F. X. Omlin. **2007.** Malaria mosquito control using edible fish in western Kenya: preliminary findings of a controlled study. **BMC Public Health**, 7: 199.

Howard A. F. V. and F. X. Omlin. **2008.** Abandoning small-scale fish farming in western Kenya leads to higher malaria vector abundance. **Acta Tropica**, 105: 67-73.

Howard A. F. V., E. A. Adongo, A. Hassanali, F. X. Omlin, A. Wanjoya, G. Zhou and J. Vulule. **2009.** Laboratory evaluation of the aqueous extract of *Azadirachta indica* (neem) wood chippings on *Anopheles gambiae* s.s. (Diptera: Culicidae) mosquitoes. **Journal of Medical Entomology**, 46: 107-114.

Howard A. F. V., C. J. M. Koenraadt, M. Farenhorst, B. G. J. Knols, and W. Takken. **2010.** Pyrethroid resistance in *Anopheles gambiae* leads to increased susceptibility to the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*. **Malaria Journal,** 9: 168

M. Farenhorst, B. G. J. Knols, M. B. Thomas, **A. F.V. Howard**, W. Takken, M. Rowland and R. N'Guessan. **2010.** Synergy in efficacy of fungal entomopathogens and permethrin against West African insecticide-resistant *Anopheles gambiae* mosquitoes. **PLoS One**, 5. e12081

Submitted

Howard A. F. V., E. A. Adongo, J. Vulule and J. Githure. Effects of a botanical larvicide derived from *Azadirachta indica* (the neem tree) on oviposition behaviour in *Anopheles gambiae s.s.* mosquitoes. Under Review

Howard A. F. V., R. N'Guessan, C. J. M. Koenraadt, A. Asidi, M. Farenhorst, M. Akogbeto, M. B. Thomas, B. G. J. Knols and W. Takken. The entomopathogenic fungus *Beauveria bassiana* reduces instantaneous blood feeding in wild insecticide-resistant mosquitoes in Benin, West Africa. Under Review

To be submitted

Howard A. F. V., R. N'Guessan, C. J. M. Koenraadt, A. Asidi, M. Farenhorst, M. Akogbeto, M. B. Thomas, B. G. J. Knols and W. Takken. The first report of the infection of insecticide-resistant malaria vectors with entomopathogenic fungi under field conditions. In Preparation

Howard A. F. V. and H. Van den Berg. Malaria vector control options available to rural African communities in the context of an integrated vector management strategy: a review. In Preparation

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Malaria vector options available to rural African communities in the context of an integrated vector management strategy: a review (2010)

Writing of project proposal (4.5 ECTS)

- Sustainable and natural mosquito control (2009)

Post-graduate courses (2.6 ECTS)

- Consumer resource interactions (2010)
- Generalized linear models (2010)

Laboratory training and working visits (4.5 ECTS)

- Experimental hut trial with entomopathogenic fungi; Centre de Recherché Entomologique de Cotonou (CREC), Benin (2009)

Invited review of (unpublished) journal (2 ECTS)

- Biological control of mosquitoes; Journal of Medical Entomology (2007)
- Botanical larvicides; Journal of Medical Entomology (2009)

Deficiency, refresh, brush-up courses (1.5 ECTS)

- Basic statistics (2010)

Competence strengthening / skills courses (3.5 ECTS)

- Presentations skills (2010)
- Mobilising your scientific network (2010)
- What you need to know to be effective in publishing your work (2010)

PE&RC Annual meetings, seminars and the PE&RC weekend (0.3 ECTS)

- On the origin of communication (2009)

Discussion groups / local seminars / other scientific meetings (4.8 ECTS)

- icipe scientist's discussion groups (2005-2007)
- Annual Meeting of the Netherlands Entomological Society (2008)
- WUR PhD student's discussion group; Entomology (2009)

International symposia, workshops and conferences (8.4 ECTS)

- The Royal Society of Tropical Medicine and Hygiene Research in Progress Meeting; London, UK (2005)
- The Royal Society of Tropical Medicine and Hygiene Research in Progress Meeting; Liverpool, UK (2006)
- 3rd KeNAAM Fresh Air Conference; Nairobi, Kenya (2006)
- 5th Biennial AMANET Conference; Zanzibar, Tanzania (2007)

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